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The structure and function of conjunctiva-associated lymphoid tissue in chickens and turkeys

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The structure and function of conjunctiva-associated lymphoid tissue in chickens and turkeys

by

Andrew Stephen Fix

A Dissertation Submitted to the
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GENERAL INTRODUCTION

Avian immunology has advanced considerably since Glick initially suggested an immunologic role for the bursa of Fabricius in 1956.[84] Since that time, many aspects of the avian immune system have been researched and clarified. These include immunoglobulin types,[103,104] histocompatibility antigens,[185] cellular cooperativity,[115,134] lymphocyte surface immunoglobulin isotypes,[3,4] and lymphoid tissues.[16,27,191] One of the most extensively studied avian lymphoid tissues is the Harderian gland.[14,163,199,200] This retrobulbar gland has been shown to contain IgA-secreting plasma cells and to have an important role in local ocular immunity.[5,41,180] Application of antigen to the conjunctival space leads to an increase in the number of plasma cells in the Harderian gland as well as the appearance of specific antibody in tears.[61,153,181] However, in spite of the marked interest in the Harderian gland, the site for initial lymphocyte contact with antigen has not been established. The only attempt at determining particulate material uptake by the Harderian gland failed to document transport across the glandular epithelium.[42]

The mucosal immune system is important for defense against potential mucosal pathogens. This is accomplished primarily through the production of secretory IgA,[22,186,189] which arises relatively late in phylogeny and ontogeny.[87] Mucosal lymphoid tissues are central to the function of the mucosal immune system. These tissues, which exist along many epithelial surfaces, are important in the uptake of antigen and in the generation of an immune response.[22,23] Many specialized structural features of mucosal lymphoid tissues have been reported, including a flattened superficial lymphoepithelium and intraepithelial lymphocytes. Particularly well-studied in this group are gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue

(BALT).[20,24,30] Although most of these studies have been restricted to mammals, some characterization of GALT and BALT has been done in avian species.[16,70,191] More recently, similar lymphoid tissue has been described in the conjunctiva of rabbits and guinea pigs.[13,47,78] This conjunctiva-associated lymphoid tissue, or CALT, has not been described or studied in avian species.

Many experiments using microorganisms or particulate tracer materials have documented the capacity for uptake of particles by mammalian GALT and BALT.[106,140,141,183] Here, uptake usually occurs by specialized mucosal lymphoepithelium through selective pinocytosis at the cell surface. The only study of experimental uptake by CALT has been done in guinea pigs.[178] In that report, uptake was demonstrated but it was not selective for the CALT lymphoepithelium.

The poultry industry relies heavily on vaccination for the prevention and control of many potentially devastating infectious diseases. Economic constraints have dictated the delivery of many of these vaccines to large numbers of birds in water or by aerosolization. Several vaccines are also delivered by eyedrop inoculation. These vaccine exposure methods make respiratory, gastrointestinal, and ocular lymphoid tissues important in the successful development of an immune response. Logically, any lymphoid tissue present within the conjunctiva that experiences vaccine contact could also be important in mucosal immunity.

Objectives of the dissertation

The objectives of these studies in turkeys and chickens were (1) to describe the structure of CALT, (2) to characterize the development of CALT during the post-hatching period, (3) to examine the response of CALT to local antigenic stimulation, (4) to demonstrate and characterize the uptake of

particulate tracers by CALT, and (5) to quantitate tracer uptake by CALT.

Explanation of dissertation format

This dissertation is presented in the alternate thesis format. It contains four manuscripts presented in the style of the journal, Veterinary Pathology. The manuscripts are preceded by a general introduction and literature review and are followed by a general summary and the literature cited. All citations for all parts are located in the literature cited section. The Ph.D. candidate, Andrew S. Fix, was the principal investigator for each study.

LITERATURE REVIEW

Mucosal membranes are epithelial surfaces that line many regions of the body, such as the gastrointestinal and respiratory tracts. The location of these epithelial surfaces places them in direct contact with the external environment. Accordingly, the ability to develop a protective immune response toward potentially pathogenic microorganisms along these epithelial surfaces is important. Mucosal lymphoid tissues are located along these surfaces specifically for this purpose. In these tissues, antigen contact and subsequent antigen uptake are important early steps in the mucosal immune response. This review addresses the pertinent literature in three areas: an initial discussion of avian and mammalian mucosal lymphoid tissues, a consideration of avian immunology, and a final discussion focused on avian paraocular immunity.

Mucosal immunity and mucosal lymphoid tissues

Mucosal lymphoid tissues, which are present along many mucosal surfaces of mammals and birds, have been studied and characterized in a number of species. In the past several decades, considerable interest has developed in the structural and functional significance of these mucosa-associated lymphoid tissues, or MALT.[21] In spite of their location in many different sites, MALT are morphologically and functionally similar. Major components of this system exist in the gastrointestinal and respiratory tracts. In addition, similar tissues have been described along several other mucosal surfaces. In the first part of this brief review, a discussion of the general structural features of MALT will be followed by a consideration of the immunologic function of these tissues.

Gut-associated lymphoid tissues, or GALT, are present throughout the gastrointestinal tract in mammals and birds.[24] GALT represents a substantial part of the total lymphoid tissue present in the body. GALT includes lymphoid tissues in the oral cavity, pharynx, small intestine, cecum, colon, bursa of Fabricius, and rectum.[146] In many species, aggregates of submucosal lymphoid tissue encircle the entrance to the digestive tract in the form of pharyngeal tonsils.[146] In pigs, palatine tonsils are exceptionally prominent in the soft palate and provide a relatively large surface area.[201] Particularly well-studied are the small intestinal Peyer's patches, which, in humans, increase in size and number from early fetal life to puberty.[37,57,144] The number of these patches in the small intestine, as well as the density of lymphoid follicles within a given patch, is highly variable between species.[146] In ruminants, both jejunal and ileal Peyer's patches exist. Like the bursa of Fabricius, ileal patches involute at maturity and have a primary function in B-cell generation; jejunal patches remain throughout life and function as secondary reactive lymphoid tissues.[98,99,156-158] Peyer's patches are also well-characterized in pigs[51,52,188] and chickens.[16] Lymphoglandular complexes, which are submucosal epithelial diverticula associated with lymphoid tissue below the muscularis mucosa, have been characterized in the large intestine of cattle,[109,110] pigs,[121] dogs,[12] and humans.[133] Lastly, the appendix of rabbits and humans contains abundant lymphoid tissue and is an important component of GALT in those species.[146,147] In rabbits, the appendix has surface recesses that delineate lymphoid nodules and which harbor luminal bacteria.[34]

Bronchus-associated lymphoid tissue, or BALT, is another important mucosal lymphoid tissue. Originally, BALT was identified along the bronchial epithelium in rabbits, guinea pigs, rats, mice, dogs, pigs, chickens, and humans.[27]

Subsequent studies have examined BALT in rats,[74] rabbits,[26,154] cattle,[9] and turkeys.[70,191] Mammalian BALT is located below bronchial epithelium and contains a narrow neck of lymphocytes connecting subepithelial lymphocytes to deeper follicles.[20,27,30] In contrast, avian BALT forms prominent subepithelial lymphoid nodules that elevate the epithelium and extend well into the lumen of pulmonary bronchi.[27,70,191]

Several other mucosal surfaces have also been found to contain regional lymphoid tissue, including reproductive, nasopharyngeal, and conjunctival. Lymphoid tissues resembling other MALT exist in the lateral walls of the mouse nasopharynx,[127] in salivary gland ducts,[126] and in the human fallopian tube and uterus.[122,197] The latter tissue apparently undergoes cyclical shedding during the menstrual cycle. More pertinent to this review are descriptions of conjunctiva-associated lymphoid tissue, or CALT.

The original descriptions of CALT were in laboratory rabbits and guinea pigs.[13,47] These reports describe randomly scattered to discretely clustered macroscopic lymphoid nodules, with a diameter of 0.2 mm to 0.8 mm, in palpebral conjunctiva. In the rabbit, these nodules are common near the nasolacrimal duct opening, average 40 per lower eyelid and 19 per upper eyelid, and are not evident before 4 weeks of age. Microscopically, CALT in rabbits consists of dense lymphoid infiltrates without germinal centers and plasma cells. In addition, lymphocytes heavily infiltrate the overlying epithelium and fill large adjacent lymphatic channels. Later study of CALT in rabbits supported the original findings but noted the presence of germinal centers and a lack of goblet cells in the overlying CALT epithelium.[78] Based on structural similarities, these studies suggested that CALT is a component of the mucosal immune system.[13,47,78] Limited work has been done with CALT since these original descriptions,[85,178] and CALT has not been described in avian species.

Mucosal lymphoid tissues exhibit remarkably similar structure, despite their diverse anatomic locations among different species. Specific divisions of these tissues include the epithelium, the lymphoid component, and the vasculature. Since the most complete morphologic descriptions of MALT have been generated through studies of GALT and BALT, these tissues provide the basis for our understanding of mucosal lymphoid tissue organization.

Perhaps the most unique division of MALT is the surface epithelium, commonly referred to as follicle-associated epithelium or lymphoepithelium. This epithelium is usually devoid of goblet cells, and it is heavily infiltrated by intraepithelial lymphocytes primarily of the suppressor/cytotoxic phenotype.[21,24] Lymphoepithelial cells typically have short microvilli, few lysosomes, flattened apical cytoplasm, abundant apical vesicles, and reduced affinity for ruthenium red.[202] Originally, cells composing the epithelium over human Peyer's patches were termed "M" cells, based on their lack of microvilli and the presence of unique surface microfolds.[144] These cells encase lymphocytes under a thin apical cytoplasm and are specialized for the uptake and transport of luminal material.[68,144,202] In addition to the general features of lymphoepithelial cells listed above, M cells have increased cholesterol content, stain poorly for alkaline phosphatase but intensely for esterase, and develop directly from undifferentiated crypt epithelial cells.[46,112,142,143] M cells have been identified in the GALT of chickens, rabbits, pigs, calves, mice, rats, guinea pigs, monkeys, hamsters, and dogs.[68,202] Similar cells exist in other mucosal lymphoid tissues including BALT[30] and CALT.[78]

A second major division of MALT, the lymphoid component, contains cells similar in identity and organization to nonmucosal lymphoid tissues. Below the lymphoepithelium are solitary or clustered lymphoid nodules composed of

lymphocytes, macrophages, reticular stromal cells, and germinal centers. Germinal centers contain expanding clones of B-cells generally committed to IgA production.[186] They are common throughout GALT, but are infrequent in BALT, except in chickens.[20,30,147] As in lymph nodes, paracortical areas are populated by T cells. The greatest number of T-helper cells are thought to be in the paracortex, whereas more T-suppressor cells exist primarily within the lymphoepithelium.[20,24] Macrophages are also distributed throughout the cellular areas of mucosal lymphoid tissues. These macrophages are important for the presentation of antigen and are positive for major histocompatibility class II antigens.[24] Recently, mucosal mast cells, which differ in several respects from their connective tissue counterparts, have been examined for their possible role in mucosal immunity.[21,30]

A third important division of MALT is the vasculature, specifically the high endothelial venules, or HEV. These specialized vessels are adapted for the homing and recirculation of lymphocytes into lymphoid tissues.[204] Such vessels are not confined to MALT, but also exist in other lymphoid tissues including lymph nodes and spleen. HEV, also called cuboidal endothelial venules or post-capillary venules, have plump, round to oval endothelial cells that closely pack the circumference of the vessel.[120] A complete phylogenetic examination of vertebrate lymphoid tissue described HEV in the lymph node, appendix, Peyer's patch, and tonsil of mammals, but only in the spleen of birds, reptiles, amphibians, and fish.[120] More recently, similar vessels have been identified in BALT of rats,[193] guinea pigs,[193] and turkeys.[70] HEV also exist in chicken GALT,[16] although no direct analogy to mammalian HEV was made. In the original reports of CALT in rabbits and guinea pigs, no specific description of HEV was made.[13,47,78]

Any discussion of mucosal lymphoid tissues is incomplete without addressing potential functions in mucosal immunity. Evidence indicates that the function of MALT is rather uniform: to protect mucosal surfaces through antigen uptake and the production of secretory IgA. Although a number of excellent papers have reviewed this subject at length,[21-23,29,87] pertinent aspects considered here will include antigen uptake, secretory IgA production, and lymphocyte homing.

Antigen uptake by mucosal lymphoid tissues is a prerequisite for the successful generation of a mucosal immune response. Studies evaluating antigen uptake by MALT have concentrated on GALT, primarily using particulate (carbon, latex beads) and macromolecular (ferritin, horseradish perodoxidase) tracer materials. Uptake occurs primarily by pinocytosis along the lymphoepithelial apical surface.[36] Histologically, carbon has been demonstrated crossing the lymphoepithelium of the rabbit appendix and the mouse Peyer's patch, with subsequent localization in mesenteric lymph nodes.[86,92,93] Similar results have been shown using latex beads in rats, mice, and dogs.[106,107,198] Latex bead uptake in Peyer's patches occurs with equal frequency in conventional and germfree mice.[105] The ultrastructural details of uptake across M cells have been elucidated using horseradish peroxidase in the mouse Peyer's patch.[140,163] In these studies, tracer material was preferentially taken up within 5 minutes by M cell apical pits, transported via cytoplasmic vesicles, and released into intercellular spaces between M cells and their enfolded lymphocytes. Tracer transport in GALT is not strictly unidirectional, since horseradish peroxidase administered intravenously also localized in the lymphoepithelium of mouse Peyer's patch, rabbit appendix, and chicken bursa of Fabricius.[38] Although fewer studies have assessed uptake in BALT, several tracers (carbon, latex beads,

and horseradish peroxidase) have been shown to be taken up by BALT lymphoepithelium.[183,192] The only report evaluating uptake in CALT failed to demonstrate selective tracer transport by conjunctival lymphoepithelium in guinea pigs.[178]

A considerable portion of humoral defense along mucosal surfaces occurs through the action of secretory IgA. This immunoglobulin has an alpha heavy chain and is frequently dimerized by the binding of the J chain peptide. The dimeric configuration enhances mucosal transport.[189] Delivery of secretory IgA along mucosal surfaces requires several specific steps. Antigen-presenting cells must process and present antigenic fragments to immature B cells.[100] In mucosal lymphoid tissues, B immunoblasts are largely committed to IgA production.[58,91] Evidence indicates that, at least in GALT, the preferential secretion of IgA is mediated by specific T cell subsets which evoke an isotype switch in B immunoblasts.[18] Similar isotype switching may also occur in guinea pig CALT.[85] Once sensitized to antigen, IgA-committed precursors leave the lymphoid tissue of origin and localize to mucosal surfaces as IgA-producing plasma cells. This localization is dependent on a number of variables, including chemotactic factors, cell receptors, organ characteristics, macrophage distribution, and vascular features.[19,59] Delivery of IgA across the epithelium requires binding of polymeric IgA to secretory component, and subsequent transport through the interior of the epithelial cell.[49,160,175,187] Secretory component, a polypeptide molecule containing five immunoglobulin-like domains, binds to IgA and is subsequently cleaved from the mucosal transport receptor located baso-laterally on the epithelial cell.[49,123,189] Secretory IgA is then released onto the adjacent mucosal surface. Secretory component has been identified in several epithelial types, including rabbit and human lacrimal glands.[7,76,77]

Selective localization of circulating lymphocytes to various mucosal lymphoid tissues is an important aspect of mucosal immunity. This subject has been extensively reviewed.[19,45,89,204] Lymphocyte recirculation patterns depend on the tissue of origin of the lymphoblast. The idea that lymphoblasts home to their tissue of origin has produced a conceptual separation of mucosal and nonmucosal immunity. Experimentally, lymphocytes derived from GALT home to the gastrointestinal tract wall or Peyer's patches, whereas cells derived from peripheral lymph nodes return to those nodes.[45] Using frozen sections of lymph nodes in an *in vitro* binding assay, several studies have clarified the role of high endothelial venules (HEV) as binding sites for the entry of lymphocytes into lymph nodes.[44,176,177] Circulating lymphocytes, which normally have a relatively smooth surface, develop microvilli as they pass through the HEV of lymph nodes.[194] The association between circulating lymphocytes and HEV is mediated by lymphocyte surface adhesion molecules that recognize specific receptors on HEV.[204] After binding, lymphocytes migrate through the endothelium and enter the surrounding lymphoid tissue. It is likely that different adhesion molecules dictate the homing of lymphocytes to different tissues.[50] Homing can be specifically confined to certain mucosal lymphoid tissues. For example, lymphocytes derived from bronchial lymph nodes localize more to the lungs than to the gut.[117] However, homing is not always predictable or completely tissue specific. In mice, ^{3}H-thymidine-labelled lymphocytes from the mesenteric lymph node preferentially repopulate multiple mucosal sites, including the gastrointestinal tract, uterus, and mammary gland.[117] In BALT of rats and guinea pigs, HEV specificity more closely resembles the HEV of mesenteric nodes than Peyer's patches.[193] There is also evidence that lymphocyte recirculation accounts for the immunological connection between the gastrointestinal

tract and the mammary gland.[89] However, the significance of this connection apparently varies with species.[88,90,167]

Avian immunology

Research in avian immunology has provided considerable insight into understanding the overall function of the immune system. Recently, many excellent reviews of this subject have been published in textbooks[67,82,149] and in journals.[56,79,81,184] Although much of the avian immune system resembles that of mammals, some unique features exist, including the bursa of Fabricius, the Harderian gland, and the lack of peripheral lymph nodes. As in mammals, avian lymphoid tissues can be divided into primary (central) and secondary (peripheral), each with a special function. These tissues contain the cells that provide the humoral and cell-mediated immunity for immunologic protection in birds. Avian primary lymphoid organs include the thymus and bursa of Fabricius, which facilitate the maturation and clonal expansion of T and B cells, respectively. Primary lymphoid tissues are also referred to as lymphoepithelial, since their lymphocytes develop in close association with an epithelial component. Secondary lymphoid tissues have an important stromal component, and are also called lymphoreticular. They are more diffuse than the primary tissues and are important in direct response to antigen.

The avian thymus, the maturation center for T cells, develops from pharyngeal pouch epithelium. It is divided into 12 to 14 distinct lobes, each containing lobules with an outer cortex and an inner medulla. A vascular barrier to antigen, the blood-thymic barrier, exists in the cortex but not in the medulla. Cell types present in the thymus include lymphocytes, epithelial cells, myoid cells, macrophages, and endothelial cells of HEV. Unlike the mammalian thymus, the

avian thymus also has a secondary immunologic function. Germinal centers exist in the medulla, where the vascular barrier is incomplete. Shortly after hatching, B cells migrate into this region and plasma cells are evident there by 4 weeks of age.[4,205]

The bursa of Fabricius, central to the generation of B cells, develops from the dorsal epithelium of the primitive cloaca.[80] Developing epithelial cells form infoldings and plicae which are colonized by lymphoid cells that subsequently define cortical and medullary areas.[1] Proliferation of these colonizing cells produces bursal follicles, which total between 8,000 and 12,000 per bursa.[136] The epithelium over these follicles develops the classic features of a lymphoepithelium specialized for antigen uptake.[35,36] However, the interfollicular epithelium lacks this specialization. Tracer particle solutions placed in contact with bursal lymphoepithelium are transported by pinocytosis. After uptake, tracers localize in bursal follicles and are distributed to other regions of the bursa by phagocytic cells.[15,129,130,174] Like the avian thymus, the bursa also has a secondary lymphoid function. Uptake by the bursal lymphoepithelium is important in this role. After local immunization, antibody-producing cells have been observed inside the bursa.[195] T cells, identified histochemically by acid alpha-naphthyl acetate staining, are also present in specific regions of the bursa.[134] In chickens, the bursa decreases in weight from 10 to 16 weeks of age, with involution virtually complete by 24 weeks of age.[131]

Secondary lymphoid tissues in birds, including the spleen, atypical lymph nodes, and mucosal lymphoid tissues, are not as well studied as in mammals. The spleen is similar to the mammalian counterpart, except for the presence of ellipsoidal sheaths of reticular cells around penicillary arteries.[137] When challenged with intravenous antigen, B and T

cell areas in the spleen cooperate and produce germinal centers in the periarterial lymphoid tissue areas.[185] The generation of splenic germinal centers is maximal at 4 to 5 weeks of age, which corresponds to peak bursal development.

Organized peripheral lymph nodes are not characteristic features of avian secondary lymphoid tissues. Instead, birds form regional concentrations of lymphoid tissue locally in organs, tissues, and lymphatic vessels.[67] However, structures resembling lymph nodes have been described. Chickens have lymphoid accumulations along the posterior tibio-popliteal and lower femoral veins.[138] These femoral lymph nodules have afferent and efferent lymphatics, peripheral germinal centers, and an intricate internal sinus system. Although barely detectable in normal birds, these structures become hyperplastic after local antigen delivery.[116] Ducks have similar paired nodes in the lumbar region that contain macrophages and vessels resembling HEV.[101] In addition, the pineal gland in chickens also contains lymphoid tissue and antibody-producing plasma cells.[53,54]

Mucosal lymphoid tissues, primarily GALT and BALT, comprise another group of secondary lymphoid tissues in birds. Peyer's patches, the cecal tonsil, and Meckel's diverticulum occur along the gastrointestinal tract. In chickens, the number of Peyer's patches increases until 16 weeks of age, then decreases until a single residual patch remains at the ileocecal junction.[16] Structurally, avian Peyer's patches resemble those in mammals.[16] Chickens also possess cecal tonsils in the proximal region of each cecum.[83] Like the mammalian palatine tonsil, these structures have epithelial crypts that are continually exposed to luminal contents. Equal numbers of B and T cells exist in cecal tonsils in chickens at 5 weeks of age; however, B cells predominate in older birds.[4] Meckel's diverticulum, the embryologic yolk sac remnant that separates the jejunum from the ileum, is

infiltrated with lymphoid cells by 2 weeks of age.[139] Like the Harderian gland, this area contains large numbers of plasma cells and remains functional until at least 20 weeks of age.[139] Ducks have a rather unique form of GALT. Annular bands of lymphoid tissue completely encircle the intestinal wall at regular intervals on either side of Meckel's diverticulum.[118] These bands are only rudimentary at hatching, but develop prominent follicles with germinal centers by maturity.

Although not mentioned in recent reviews of avian immunology,[67,82,149] BALT was characterized in chickens almost two decades ago.[27,28] In those studies, lymphoid nodules with germinal centers formed finger-like projections into the bronchial lumen. According to the authors, chickens had more lymphoid tissue in the trachea than any other species examined (rabbit, guinea pig, rat, mouse, dog, pig, human). Their use of the term "trachea" is somewhat perplexing, since by definition, BALT is confined to the pulmonary bronchus. Subsequent and more complete descriptions of BALT have been reported in turkeys.[70,191] In this species, BALT nodules protrude into the primary intrapulmonary bronchus and are specifically associated with longitudinal mucosal folds and the openings of secondary bronchi.[70] This study also demonstrated a progressive increase in the number and size of BALT nodules with increasing age of turkeys. In another study that involved turkeys infected with Bordetella avium, BALT nodules were more numerous and more widely distributed than in normal birds.[191] In contrast to mammals,[183] specific experiments documenting tracer uptake have not been done in avian BALT. Although the ultrastructural features of turkey BALT lymphoepithelium does not suggest specialization for antigen uptake, epithelial discontinuities induced by infiltrating lymphocytes may allow antigens to pass from the lumen to underlying lymphoid elements.[70]

Several studies have localized antibody-producing cells in avian mucosal lymphoid tissues by immunohistochemistry. In the chicken gastrointestinal tract, IgA-positive cells have been identified in the duodenum and cecal tonsil, but not in Peyer's patches.[25] This has been verified by some reports,[102] but not by others.[10] The latter study found primarily IgG-positive cells along the chicken gastrointestinal tract. In turkey intestine, IgG-positive cells are sparse and IgA-positive cells equal those positive for IgM.[55] Antibiotics reduce the number of immunoglobulin-bearing cells in the gastrointestinal tract of both chickens and turkeys.[55,128] Along chicken bronchial mucosa, IgG- and IgM-positive cells occur most commonly, with fewer IgA-positive cells evident.[10,25]

Humoral and cellular aspects of avian immunology are well characterized. Three major immunoglobulins occur in avian serum, a 7S immunoglobulin analogous to mammalian IgG, IgM, and an IgA-like immunoglobulin.[17] Serum IgG and IgM are present in highest concentration, with IgA representing less than 4% of total serum immunoglobulins.[104] Only 20% of serum IgA is monomeric, with the remainder existing as dimers or multimers.[104] In chickens, IgA predominates in bile and intestinal secretions,[104,162,168] but not in saliva, tears, or seminal plasma.[104] In contrast, IgG predominates in tracheal secretions.[48] The transport of biliary IgA has been extensively studied in chickens, and a molecule similar to mammalian secretory component is likely involved.[31,152,196] In the chicken egg, IgG is concentrated in the yolk and IgM and IgA are confined principally to the white.[161] Similar serum and secretory immunoglobulin profiles exist in turkeys, with IgG and IgM detectable in many secretions.[165] Polymeric IgA occurs in the bile and intestinal secretions of turkeys,[111] with some IgA also detectable in saliva, tears, and trachea.[65]

Although IgM and IgA are present in the turkey egg, IgG is the predominant immunoglobulin found.[64]

Several aspects of cellular immunity have been studied in birds and many functional types of T cells have been identified. Chickens have T helper and T suppressor cells that have similar functions to their mammalian counterparts.[32,79,115] T suppressor cells are bursal dependent, since bursectomized chickens demonstrate reduced suppressor activity.[66] In contrast, bursectomy does not reduce cytotoxic T cell function.[96] Delayed-type hypersensitivity reactions occur in chicks exposed to tuberculin, diphtheria toxoid, and human gamma-globulin.[82] Contact hypersensitivity reactions to topically administered low molecular weight compounds are modulated by B cells.[149] Other cellular aspects of avian immunology that have been explored include homograft and allograft rejection, graft-versus-host responses, and the major histocompatibility complex.[56,82]

Avian paraocular anatomy and immunity

The general structural features of the tissues and organs surrounding the avian eye are presented in several recent textbooks of avian anatomy.[69,94,95,132] However, one of the earliest and most thorough accounts was in the English sparrow in 1918 by Slonaker.[173] Birds have a closed, bony orbit that contains the eyeball, paraocular glands, extraocular muscles, nerves, blood vessels, fat, and connective tissue. A nicitating membrane, or third eyelid, exists in the rostral angle of the conjunctival space. For lubrication and cleaning of the cornea, this thin fibrous membrane is rapidly swept across the cornea by a tendon that connects to a specific retrobulbar muscle, the pyramidalis. Paraocular glands present in birds include the lacrimal gland and the Harderian gland. The lacrimal gland, which contributes to tear

production, is located at the lateral orbital rim and drains into the conjunctiva by several small ducts. The Harderian gland is a retrobulbar, tubulo-alveolar gland that also contributes to tear secretions. It empties into the conjunctival space behind the nictitating membrane through a single duct. To cover and protect the cornea, birds have upper and lower eyelids like mammals. However, detailed descriptions of these structures are lacking in avian anatomy texts and journals.

Considerable scientific literature exists delineating the role of the Harderian gland in avian paraocular immunity. Although the existence of the gland was known for centuries, in 1968 Bang and Bang described "invasive lymphoid infiltrates" along the lacrimal gland, the Harderian gland, the ducts of those glands, and the duct system of the lateral nasal gland.[14] These workers emphasized that infiltrates along the ducts were primarily small lymphocyte nodules with germinal centers. In contrast, the Harderian gland was infiltrated with large populations of plasma cells. Subsequent studies have verified and extended the original findings in chickens,[97,163,199,200] and have added descriptions of the Harderian gland in turkeys[43,114] and ducks.[39,43,114] In addition, plasma cells exist in the Harderian gland of many wild bird species,[40] and have also been found in germfree chickens.[14] Some reports have emphasized the large number of Russell bodies in Harderian gland plasma cells.[199]

Morphologically, the Harderian gland has the following features: a compound tubulo-acinar architecture with lobules, columnar epithelium, fibrovascular interstitial septae, and a thin connective tissue capsule.[199] Histochemically, the merocrine secretions are mostly sulfated mucopolysaccharides.[200] Four types of epithelial cells have been characterized ultrastructurally.[163] Several lobule types exist in the Harderian gland, and the relative plasma cell

density varies between types.[40] Plasma cell infiltration is interstitial, bursal dependent,[124,179] and increases with age.[180] In chickens, early infiltration occurs between 2 and 4 weeks of age, primarily with cells positive for IgM.[5] However, IgG- and IgA-positive cells predominate from weeks 4 to 9, with mostly IgA-positive cells present thereafter.[5,180] In contrast, others have found primarily IgM- and IgG-positive cells in the Harderian gland of adult chickens.[25] The gland also contains macrophages, lymphocytes, myoepithelial cells, and heterophils.[14,163,169] Macrophages, which are structurally heterogeneous, exist in both intraepithelial and subepithelial sites and sometimes are associated with dense homogenous intercellular material.[169] Ultrastructurally, pre-plasma cells or "plasmablasts" form desmosome-like junctional complexes with each other and with nearby macrophages.[169]

A number of respiratory diseases exist in birds that have considerable negative economic impact on the poultry industry. These include Newcastle disease, infectious bronchitis, avian pox, infectious laryngotracheitis, and turkey bordetellosis. Many important aspects of resistance to these respiratory pathogens have recently been reviewed.[60] In an effort to improve health and increase productivity, the poultry industry has developed many vaccines to help control these diseases. A significant number of these vaccinations require delivery on a flock basis, since individual bird handling is cost prohibitive. Therefore, administration is usually by water or aerosol. Protocols for the administration of these vaccines vary widely in the industry. Since vaccination success depends on epithelial surface contact, mucosal lymphoid tissues are undoubtedly important in the generation of protective immunity. For several decades, the avian Harderian gland has been regarded as central to the production of local immunity, especially in response to eyedrop or aerosol vaccination.

Several studies have assessed antibody production by Harderian gland plasma cells. Specific homologous antibody was detected in saline extracts of Harderian gland after conjunctival inoculation with sheep red blood cells, Newcastle disease virus, infectious bronchitis virus, and Mycoplasma gallisepticum.[153] However, no antibody was detectable in the Harderian gland after parenteral inoculation with these agents. Similar results have been demonstrated by others with conjunctival delivery of Newcastle disease virus[113,148] and bovine serum albumin.[41] Using immunofluorescence, this latter report also identified plasma cells with antibody specificity for bovine serum albumin. Studies using infectious agents have indicated that during inflammation, some circulating antibody enters Harderian gland secretions by transudation.[2]

Structural changes in the avian Harderian gland after conjunctival delivery of infectious agents have been examined in chickens. Eyedrop delivery of live Newcastle disease virus and live infectious bronchitis virus produced a significant increase in Harderian gland plasma cells; however, aerosol or intravenèous inoculation produced only a slight increase.[61,181] These studies also documented acute inflammatory changes in affected Harderian glands. After inoculation with infectious bronchitis virus, lymphoid follicles formed in the Harderian gland in 14 to 21 days.[61] An opposite effect occurred after conjunctival inoculation of infectious bursal disease virus in 3-week-old broilers, since Harderian gland plasma cells became necrotic for 5 to 14 days.[63] However, plasma cell numbers did eventually return to normal. Similar inoculation in 1-day-old broilers prevented the normal post-hatching infiltration of plasma cells into the Harderian gland.[62] Comparable effects by infectious bursal disease virus have been substantiated by others.[108,150]

In spite of the strong interest in the Harderian gland as a paraocular lymphoid organ, few attempts to document antigen

uptake by this gland have been made. After conjunctival delivery, carbon and colloidal gold tracers were found in the Harderian gland secretory duct.[42] Although these tracers were trapped within glandular secretions, no evidence of direct epithelial contact or transport were reported. The uptake of tracer particles or antigen has not been documented anywhere along paraocular surfaces or the upper respiratory tract of birds.

PART I. CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN NORMAL AND *BORDETELLA AVIUM*-INFECTED TURKEYS

CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN NORMAL AND *BORDETELLA AVIUM*-INFECTED TURKEYS

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ABSTRACT

Conjunctiva-associated lymphoid tissue (CALT) was characterized in normal and Bordetella avium-infected turkey poults during the first 5 weeks of life. At 1, 5, 12, 19, 25, and 33 days post-hatching (DPH), upper and lower eyelids were examined by gross, histologic, and electron microscopic techniques. CALT was confined to the proximal part of the lower eyelid near the conjunctival fornix; it appeared by 5 DPH as individual lymphoid nodules and as dense masses by 19 DPH. In the upper eyelid, CALT was present only as isolated nodules. Histologically, CALT was composed of dense lymphocyte infiltrates within subepithelial connective tissue, intraepithelial lymphocytes, and flattened lymphoid-associated epithelium that lacked goblet cells. Germinal centers were in CALT by 19 DPH. By scanning electron microscopy, epithelial cells over lymphoid areas were flat and had short, irregular microvilli; non-lymphoid areas were covered by cells with tall, regular microvilli. Transmission electron microscopy revealed that with increasing age of birds, the epithelium over conjunctival lymphoid infiltrates became progressively flattened and infiltrated by lymphocytes. Some blood vessels in CALT had high endothelial cells; lymphocytes were in the lumen and between or beneath endothelial cells. In B. avium-infected poults, CALT was increased, developed earlier, and contained more germinal centers than in normal poults. We conclude that CALT of turkeys closely resembles other mucosal lymphoid tissues and may serve as a site for local antigen uptake.

INTRODUCTION

Mucosal lymphoid tissues contain cells which allow antigen uptake and processing with subsequent generation of an antibody response, primarily in the form of secretory IgA.[22] Gut-associated lymphoid tissue (GALT)[16,24,146] and bronchus-associated lymphoid tissue (BALT)[20,190] have been described in both birds and mammals. The Harderian gland, which contains plasma cells[180] and secretory IgA, [5] has also been described in birds.[14,169,199] Conjunctiva-associated lymphoid tissue (CALT) is morphologically similar to GALT and BALT, but descriptions are limited to rabbits and guinea pigs.[13,47,78] CALT contains multiple nodules of lymphocytes beneath the palpebral conjunctiva of the lower eyelid.[13,47] Unlike CALT, the avian Harderian gland has little opportunity for antigen contact since it is confined to the orbit and is not exposed to the conjunctival surface. It is possible that CALT functions as a site for initial antigen contact with subsequent distribution of antibody-producing cells to mucosal surfaces and secretory glands.[13,47,78]

CALT might be expected to undergo some degree of hyperplasia if subjected to a local antigenic challenge, such as that which accompanies an infection. Bordetella avium is the cause of turkey bordetellosis (coryza), a highly contagious upper respiratory tract disease of young turkeys characterized by oculonasal discharge, sneezing, and conjunctivitis.[166,172] Turkeys infected with B. avium have hyperplastic peribronchial lymphocytic nodules (BALT), probably in response to sustained antigenic stimulation associated with infection.[190] Similarly, the conjunctivitis associated with B. avium infection[172] might provide antigenic exposure to lymphoid tissue present within the conjunctiva. The specific objectives of the present study were to describe anatomical features of the turkey eyelid pertinent to CALT,

characterize the development of CALT in normal turkeys, and to compare the development of CALT in normal and *B. avium*-infected turkey poults.

MATERIALS AND METHODS

Seventy-seven 1-day-old Nicholas strain Broad-Breasted White turkeys were obtained commercially (Midwest Turkey Hatchery Inc., Dike, Iowa) and divided into two groups. All turkeys were provided accessory heat, turkey starter, and water ad libitum. Turkeys in one group (n = 35) were inoculated in each eye and each nostril with 50 μl of a Bordetella avium strain 75 stock culture (6.8×10^8 colony-forming units/ml) as previously described.[11] The other group (n = 42) remained as noninoculated, normal turkeys and were housed separately from the inoculated group. At specific intervals post-hatching (5, 12, 19, 25, and 33 days), seven turkeys from each group were euthanatized after blood collection (1 ml) and the upper and lower eyelids removed with the conjunctiva intact. A group of seven noninoculated, normal birds received similar treatment at 1 day of age. For all turkeys at all intervals, serologic response to B. avium was determined with a microtiter agglutination test as previously described.[11] Eyelids were characterized morphologically by gross, histologic, scanning electron microscopic, and transmission electron microscopic techniques.

For gross observation, a procedure developed for the specific staining of gastrointestinal Peyer's patches was used.[57] Briefly, eyelids were fixed in 3% acetic acid for 24 hours, washed in water, stained with 0.5% aqueous methylene blue, and washed again in water until sufficient stain was removed from non-lymphoid areas to maximize contrast between these areas and conjunctiva-associated lymphoid tissue. Eyelids were then placed on a glass plate and studied by transillumination.

For histologic examination, eyelids were pinned on cork to prevent distortion of the conjunctival epithelium and to best approximate in vivo orientation. Following fixation in

10% neutral buffered formalin, eyelids were trimmed in cross-section perpendicular to the free lid margin, processed by routine paraffin technique, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff, and Masson's trichrome. Selected eyelids were embedded in glycol methacrylate (Bio Rad, Richmond, California), sectioned at 2 μm, and stained with 0.1% aqueous toluidine blue or HE.

Tissues for scanning electron microscopy were fixed in ice-cold (4 C) 3% glutaraldehyde and 8% tannic acid in 0.05 M Sorensen's phosphate buffer, pH 7.3. To enhance conductivity, specimens were further processed in tannic acid and osmium tetroxide as previously described.[182] Tissues were rinsed in deionized water, dehydrated in ethanol and Freon 113, critical point dried from carbon dioxide, mounted on aluminum stubs, sputter-coated with gold-palladium for 2.5 minutes, and examined with a Cambridge Stereoscan 200 scanning electron microscope.

For transmission electron microscopy, tissues were fixed in 3% glutaraldehyde in 0.05 M Sorensen's phosphate buffer, pH 7.3, and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in alcohol and embedding in epoxy resin (EMbed 812, Electron Microscopy Sciences, Fort Washington, Pennsylvania), semi-thin sections were examined by light microscopy. Ultrathin sections were cut and stained with 2% methanolic uranyl acetate and Reynolds' lead citrate and examined with a Hitachi HS-9 transmission electron microscope.

RESULTS

All Bordetella avium-infected turkeys developed clinical signs typical of bordetellosis including sneezing, oculonasal discharge, and conjunctivitis. The noninoculated group remained clinically normal throughout the post-hatching period. In the inoculated group, serologic response to B. avium infection was detected by the microtiter agglutination test at 12 days post-hatching (DPH) and peaked by 25 DPH. Normal, noninoculated birds consistently had no titers. Although both upper and lower eyelids were examined in this study, only an occasional isolated lymphoid nodule was present within the conjunctiva of upper eyelids. Subsequent descriptions refer only to conjunctiva-associated lymphoid tissue (CALT) within the lower eyelid (ventral palpebra).

Gross appearance of CALT

For orientation, the conjunctival surface from the ventral palpebra of a normal turkey poult (19 DPH) is presented in diagrammatic form (Fig. 1). CALT is evident as dense nodules within proximal semilunar longitudinal folds and fissures along the proximal portion of the palpebral conjunctiva, close to the conjunctival fornix. CALT is more concentrated along the nasal (rostral) margin of the eyelid than along the temporal margin. Distal linear folds and fissures, located immediately below the palpebral rim, do not contain lymphoid tissue.

In normal turkeys, the gross appearance and development of CALT followed a distinct temporal pattern in all birds examined. Although not evident at 1 DPH, CALT was seen by 5 DPH as individual nodules along proximal longitudinal folds and fissures. These nodules coalesced into aggregates by 12 DPH and formed dense masses by 19 DPH. By 33 DPH,

considerable lymphoid tissue was present beneath the conjunctival epithelium (Fig. 2a).

In B. avium-infected turkeys, the development of CALT closely resembled the pattern in normal birds. However, by 12 DPH, relatively more lymphoid tissue was evident beneath the conjunctiva of infected birds. The increase in lymphoid tissue continued to be detectable at 19 and 25 DPH and was pronounced by 33 DPH (Fig. 2b).

Light microscopy

CALT was consistently localized to longitudinal folds and fissures in the proximal part of the lower eyelid, close to the the conjunctival fornix (Fig. 3). No lymphoid tissue was present along the more distally located epithelial plateau or the longitudinal folds and fissures next to the palpebral rim.

In normal turkeys, the developmental progression of CALT in the post-hatching period had a consistent histologic pattern in all birds examined. At 1 DPH, proximal folds and fissures contained tall columnar to cuboidal epithelial cells, numerous goblet cells, and only occasional individual lymphocytes within subepithelial connective tissue. By 5 DPH, lymphocytes were numerous within the subepithelial tissue of conjunctival folds, and occasional intraepithelial lymphocytes (IEL) were evident (Fig. 4). Distinct lymphoid nodules that distended and disrupted folds and fissures were seen by 12 DPH and were prominent by 19 DPH (Fig. 5). These nodules were covered by a thin, flattened epithelium that contained numerous IEL (Fig. 6). Germinal centers were detected within lymphoid nodules by 19 DPH and were prominent by 33 DPH. These centers were characterized by large blast cells, mitotic figures, pyknotic nuclear debris within macrophages, and peripheral collagenous and reticular fibers. Multiple germinal centers per nodule were seen occasionally.

Bordetella avium-infected turkeys had histologic features of CALT development similar to normal turkeys, but lymphoid tissue was more prominent at each post-hatching interval examined. At 5 DPH, in contrast to normal turkeys (Fig. 4), early epithelial attenuation was detectable over lymphoid areas in B. avium-infected birds (Fig. 7). By 19 DPH, several infected individuals had extremely prominent, dome-shaped nodules containing multiple germinal centers. Infected birds generally had larger and more prominent germinal centers than did normal birds.

Scanning electron microscopy

In normal turkeys, differences between conjunctival epithelial cells over lymphoid and non-lymphoid areas were detected by scanning electron microscopy. In non-lymphoid areas, the region containing proximal longitudinal folds and fissures consisted of uniform, parallel, cleft-like fissures separated by folds of epithelium. Cells at the surface of folds had tall, regular microvilli, but occasional cells had long, prominent microvilli (Fig. 8). In lymphoid areas, proximal folds and fissures were distended and disrupted as infiltrating lymphocytes formed nodules and large aggregates. Epithelial cells between adjacent lymphoid nodules were densely compressed while those over lymphoid nodules had a flattened and stretched appearance (Fig. 9). Most of the epithelial cells between adjacent lymphoid nodules had uniform microvilli, but occasional cells had surfaces with more stellate projections (Fig. 10). In comparison, epithelial cells over lymphoid areas had short, regular to irregular microvilli that incompletely covered many cells (Fig. 11).

In B. avium-infected turkeys, epithelial cells over both lymphoid and non-lymphoid areas were similar to epithelial cells in normal turkeys. However, distention and disruption

of proximal folds and fissures by lymphoid nodules were more extreme in the infected birds and occurred earlier.

Transmission electron microscopy

During the post-hatching period in all turkeys, the conjunctival epithelium over lymphoid nodules became progressively flattened and infiltrated with lymphocytes. At 1 DPH, epithelial cells in proximal folds and fissures were uniform, had tall, regular microvilli and terminal junctions, and contained occasional cytoplasmic vacuoles (Fig. 12). Basal cells were more electron dense than superficial cells, contained intermediate filaments, and had a continuous, uninterrupted basement membrane. By 5 DPH, numerous solitary IEL were evident within the epithelium (Fig. 13). These IEL, which frequently breached the basement membrane, had cytoplasmic extensions that projected between and separated basal epithelial cells. Superficial epithelial cells were slightly flattened over IEL and had prominent terminal junctions. Microvilli were tall and regular. At 12 and 19 DPH epithelial flattening and IEL infiltration were more pronounced. Flattened superficial cells covered IEL and had shorter microvilli than at 5 DPH. Solitary IEL were common, but a few small IEL clusters were seen. By 25 DPH, superficial epithelial cells were extremely flattened, had infrequent short, irregular microvilli, and covered large aggregates of IEL (Fig. 14). Basal epithelial cells were compressed between IEL and contained prominent intermediate filaments. Lymphocytes typically formed distinct intraepithelial aggregates, and solitary IEL were less common than at 12 and 19 DPH. Epithelial characteristics at 33 DPH were similar to 25 DPH.

Conjunctival blood vessels in lymphoid areas, but not in non-lymphoid areas, developed high endothelium and close

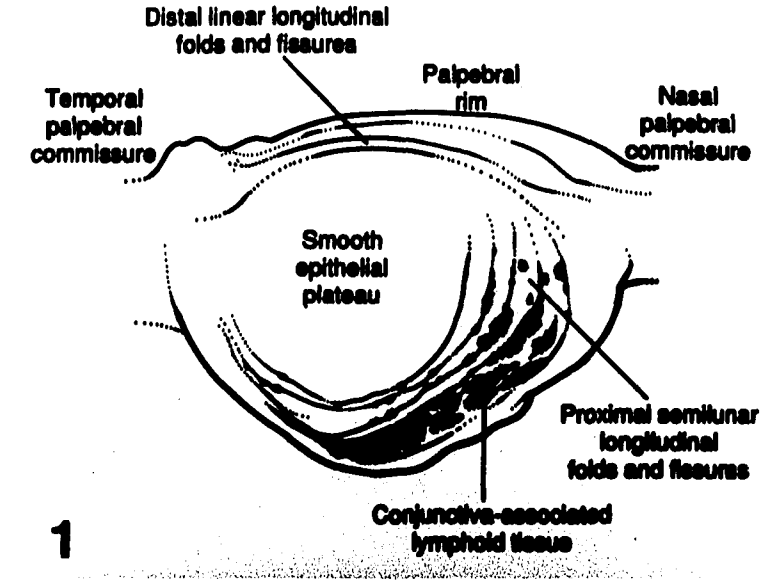
membrane association with intraluminal lymphocytes in all turkeys examined during the post-hatching period. Subepithelial capillaries and venules at 1 DPH contained thin endothelial cells and erythrocytes (Fig. 12). At 5 DPH, endothelial cells in venules were thicker, and intraluminal lymphocytes had cytoplasmic projections which contacted the endothelial surface. Tall, plump endothelial cells which projected into the venule lumen were evident by 12 DPH (Fig. 15). Lymphocytes in these venules had features similar to those in vessels at 5 DPH but were also seen beneath endothelial cells and within the vessel wall itself. Venules in lymphoid areas at 25 DPH were larger, had continuous high endothelium, and contained lymphocytes between and beneath adjacent endothelial cells. These characteristics were unique to lymphoid-associated venules and were not seen in other conjunctival venules where lymphoid nodules were lacking.

Significant differences in the ultrastructural features of CALT could not be detected between normal and B. avium-infected turkeys.

Fig. 1: Conjunctival surface of the lower eyelid, 19-day-old normal turkey poult. Conjunctiva-associated lymphoid tissue associated with proximal semilunar longitudinal folds and fissures. Distal linear longitudinal folds and fissures and smooth epithelial plateau lack lymphoid tissue

Fig. 2: Conjunctiva-associated lymphoid tissue within lower palpebral conjunctiva, 33-day-old normal (a) and Bordetella avium-infected (b) turkeys poults. Increased lymphoid tissue in the infected bird (b). Acetic acid fixation, aqueous methylene blue Bar = 3 mm

Fig. 3: Proximal half of lower eyelid, 25-day-old Bordetella avium-infected turkey poult. Conjunctiva-associated lymphoid tissue distends and disrupts proximal longitudinal folds and fissures (arrows) near fornix (1). Smooth epithelial plateau (2) lacks lymphoid tissue. Note germinal centers within lymphoid nodule (3). HE. Bar = 1 mm


Distal linear longitudinal folds and fissures
Palpebral rim
Temporal palpebral commissure
Nasal palpebral commissure
Smooth epithelial plateau
Proximal semilunar longitudinal folds and fissures
Conjunctive-associated lymphoid tissue

1

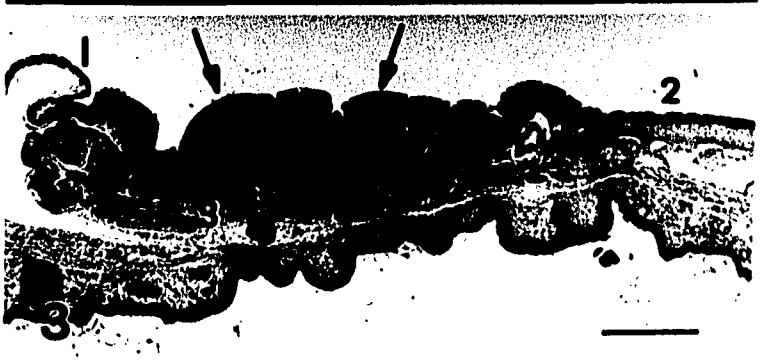

1
2
3

Fig. 4: Conjunctival fold, 5-day-old normal turkey poult. Note subepithelial lymphocytes, occasional intraepithelial lymphocytes, and limited epithelial attenuation. HE. Bar = 20 μm

Fig. 5: Lymphoid nodules distend and disrupt conjunctival folds, 19-day-old normal turkey poult. Compare tall columnar epithelial cells in non-lymphoid areas (long arrow) to flattened cells over lymphoid nodules (short arrow). Glycol methacrylate, toluidine blue. Bar = 100 μm

Fig. 6: Higher magnification of flattened epithelium over lymphoid nodules from Fig. 5. Intraepithelial lymphocyte clusters (arrowheads) between epithelial cells and above basal lamina. Glycol methacrylate, toluidine blue. Bar = 15 μm

Fig. 7: Conjunctival fold, 5-day-old Bordetella avium-infected turkey poult with numerous subepithelial lymphocytes, epithelial attenuation, and intraepithelial lymphocytes. Compare to normal bird, Fig. 4. HE. Bar = 50 μm

Fig. 8: Conjunctival epithelium from a non-lymphoid area within the lower eyelid, 5-day-old normal turkey poult. Microvilli are tall and regular (1) or long and prominent (2). Bar = 2 μm

Fig. 9: Conjunctiva-associated lymphoid epithelium, 19-day-old normal turkey poult. Compressed cells (1) located between nodules covered by flattened cells (2). Bar = 50 μm

Fig. 10: Epithelial cells between lymphoid nodules from (1) in Fig. 9. Microvilli uniform, but occasional cells have stellate projections (arrow). Bar = 2 μm

Fig. 11: Epithelial cells over lymphoid nodules from (2) in Fig. 9. Microvilli are short, irregular, and incompletely cover some cells. Bar = 5 μm

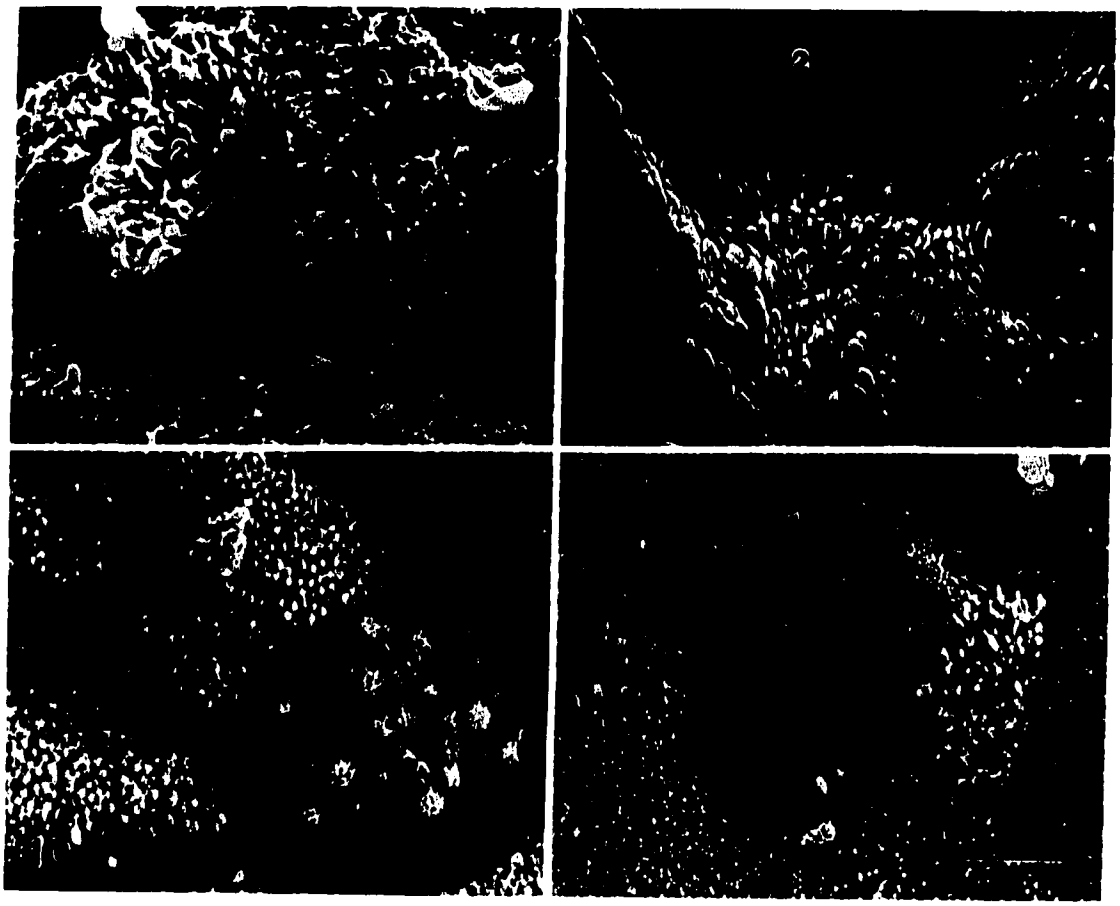


Fig. 12: Conjunctival epithelium, 1-day-old normal turkey poult. Note uniform, tall microvilli, cytoplasmic vacuoles, terminal junctions, and intermediate filaments. Erythrocyte within subepithelial capillary below basal lamina (1). Bar = 2 μm

Fig. 13: Conjunctival epithelium, 5-day-old normal turkey poult. Intraepithelial lymphocytes (1) and prominent terminal junctions (arrowheads). Bar = 2 μm

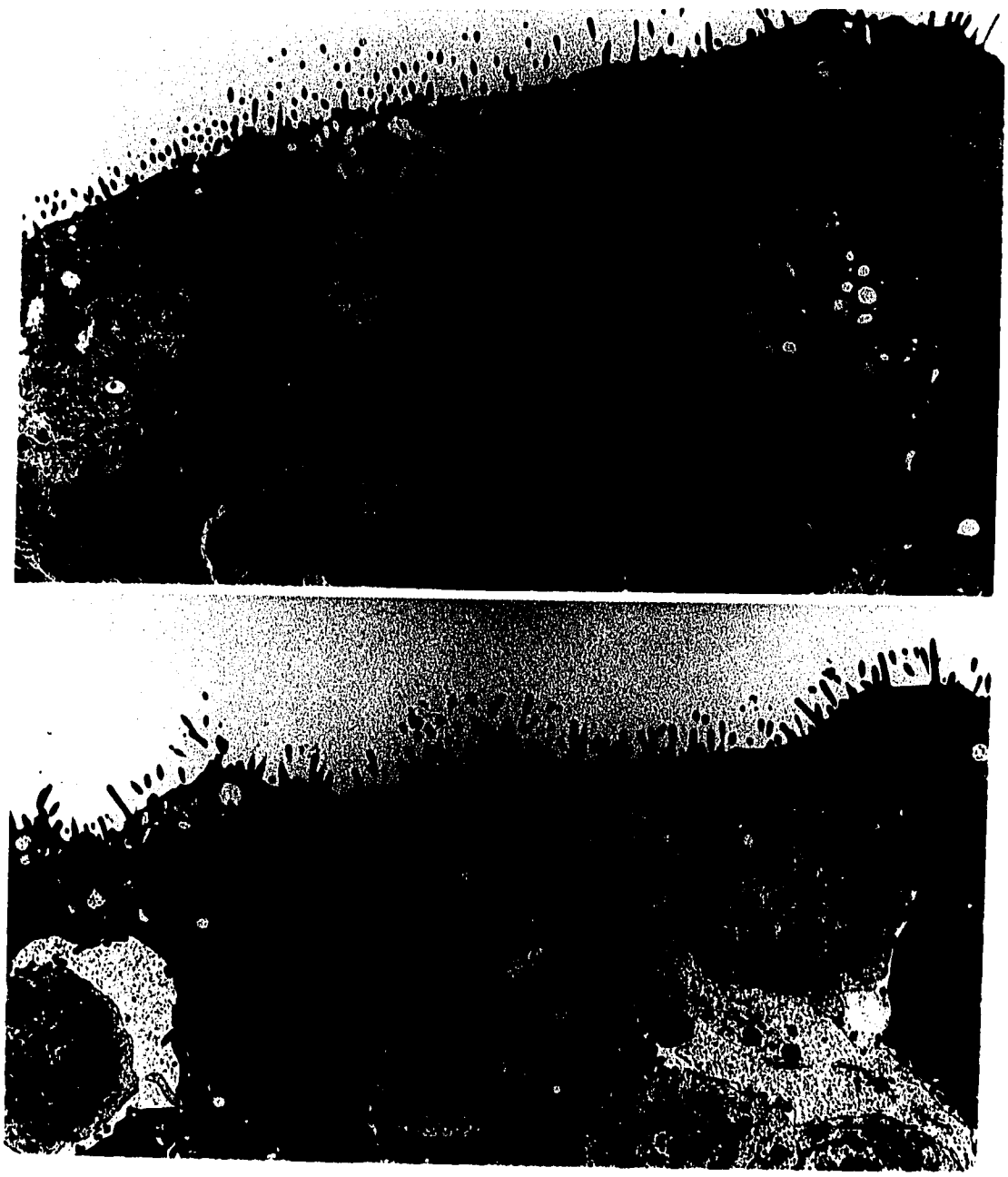


Fig. 14: Conjunctival epithelium, 25-day-old normal turkey poult. Flattened epithelial cell with short, irregular microvilli over intraepithelial lymphocyte aggregate. Note junctions with adjacent cell (arrow). Bar = 2 μm

Fig. 15: Lymphoid-associated venule, 12-day-old normal turkey poult. Lymphocytes (1) in lumen (2) associated with high endothelium by cytoplasmic processes (arrowheads). Note three subendothelial lymphocytes. Bar = 2 μm

DISCUSSION

Based on location, epithelial structure, vascular features, and cellular composition, conjunctiva-associated lymphoid tissue (CALT) of turkeys is probably important in mucosal immunity. The conjunctival location may provide exposure to environmental antigens, including microorganisms, which are routinely encountered by the bird. These antigens could gain access to responsive lymphoid cells by uptake through specialized conjunctival epithelial cells. The vessels containing high endothelium and associated lymphocytes may facilitate localization and entry of appropriate lymphocytes into the region. Although the lymphoid cell population in CALT has not been specifically characterized in turkeys, both B and T cell components are likely. Using mitogenic stimulation and immunofluorescence in rabbits, CALT has been shown to contain both T and B cells, with a large proportion of IgA-committed B cells.[78] These IgA precursor cells may undergo expansion within the blast cell areas of rabbit CALT described in previous studies.[13,47] A similar event may occur within the germinal centers found in the turkey CALT of the present study. This is particularly likely since the antigenically stimulated Bordetella avium-infected birds had more germinal centers within conjunctival lymphoid tissue. After differentiation into mature IgA-producing plasma cells, localization of these cells to various ocular surfaces is likely to provide the delivery of secretory IgA to the nearby epithelium.[75]

Similarities and differences exist between features of CALT in rabbits[13,47,78] and CALT of the turkeys in the present report. In both species, lymphoid nodules are not evident at birth or hatching, appear more common in the lower palpebra than the upper palpebra, are confined primarily to the proximal conjunctival region, and vary in distribution from

single, scattered nodules to dense aggregates.[13] Histologically, CALT in both species contains intraepithelial lymphocytes but lacks goblet cells and plasma cells.[13,47,78] However, the time of appearance of CALT differs. Lymphoid tissue is evident in turkey conjunctiva by 5 days post-hatching (DPH) but does not appear in rabbits until sometime after 4 weeks of age.[13] Rabbits also have more nodules in the upper conjunctiva than turkeys.[13] Lymphoid-associated epithelial cells in rabbit CALT have abundant, long microvilli and microplicae,[47] but in turkeys these cells have sparse, short, and irregular microvilli and lack microplicae. CALT in rabbits lacks the prominent germinal centers, significant epithelial attenuation, high endothelial venule (HEV)-like blood vessels, and specific association with conjunctival epithelial folds and fissures[13,47,78] that are characteristic in turkeys. In addition, rabbit CALT contains lymphocyte-packed lymphatic channels peripheral to lymphoid nodules that are not seen in turkeys.[13]

Epithelial and vascular features of CALT in turkeys are similar to previously characterized components of the mucosa-associated lymphoid system. The close association of lymphoid nodules with a corresponding epithelium is typical in avian and mammalian descriptions of gut-associated lymphoid tissue[16,144,146,149] and bronchus-associated lymphoid tissue.[27,190] In these reports, the lymphoid-associated epithelium is characterized by cell attenuation, fewer microvilli on epithelial cells, loss of goblet cells, and infiltration by lymphocytes. Several terms are used in the literature for this specialized epithelium, including follicle-associated epithelium and lymphoepithelium.[202] The lymphoepithelium allows selective sampling of local antigens through M cells,[144] and facilitates presentation of those antigens to nearby cells of the immune system.[20,144] The venules in turkey CALT closely resemble specialized HEV of

other mucosa-associated lymphoid tissues. HEV are characterized by cuboidal endothelium, intravascular and intramural lymphocytes, and specific cell recognition mechanisms that determine migration pathways for recirculating lymphocytes.[20,176,204] The endothelial cell-lymphocyte association in HEV occurs through cytoplasmic projections and is probably a receptor-mediated event.[176,204]

The existence of plasma cells and the associated production of secretory IgA is well documented in the paraocular glands of rabbits,[77] humans,[6,8,76] and birds.[14,82,149,199] These reports describe pure populations of interstitial plasma cells in the mammalian lacrimal gland and the avian Harderian gland, both of which are major contributors to tear production through ducts that empty into the conjunctival space. Secretory IgA is immunohistochemically detectable within glandular acini and plasma cells in these glands.[5,76,77] Additionally, secretory component, the transport molecule responsible for transepithelial delivery of secretory IgA, is also detectable in both human and rabbit lacrimal gland epithelium.[7,77] Although these reports collectively provide strong evidence for the existence of protective immune mechanisms at the ocular surface, the initial site of antigen contact is still undetermined.

CALT in rabbits has been proposed to function in early antigen contact with the subsequent generation of IgA-committed blast cells that recirculate and localize to specific mucosal surfaces.[13,47,78] Based on the morphologic features of turkey CALT presented in this study, the strong resemblance of this tissue to its counterpart in rabbits, and the documented existence of IgA in the lacrimal secretions of turkeys,[64] we propose that CALT in turkeys functions as an initial site of antigen uptake. Processing of antigen and presentation to immature lymphocytes might cause these cells

to differentiate into IgA-producing plasma cells that home to mucosal surfaces and secrete IgA for transport across local epithelial surfaces. Although these cells may home to any mucosal surface, the large population of plasma cells in the Harderian gland of turkeys[169,180] suggests that this gland is important for antibody delivery into ocular secretions. Since these secretions also contact the upper respiratory passages via the naso-lacrimal duct, antibody from the Harderian gland can potentially contact a very large mucosal surface. Initial antigenic exposure via CALT, with subsequent localization of plasma cells to the Harderian gland or nearby epithelial tissues, may provide a homing-loop mechanism specific for the protection of ocular and upper respiratory surfaces in turkeys. If this proves to be true, protection against significant upper respiratory diseases may be achieved or enhanced through the use of eyedrop and aerosolized vaccines.

PART II. MORPHOLOGIC CHARACTERIZATION OF CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN CHICKENS

MORPHOLOGIC CHARACTERIZATION OF CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN CHICKENS

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ABSTRACT

Conjunctiva-associated lymphoid tissue (CALT) in the eyelids of chickens was studied by gross, histologic, and electron microscopic techniques. Structural features were characterized at 1 day of age and at 1, 2, 3, 4, 6, 8, 12, and 16 weeks post-hatching (WPH). Beginning at 1 WPH, prominent lymphoid nodules containing a heterogenous population of lymphocytes, lymphoblasts, and macrophages were first observed within conjunctival folds and fissures of the lower eyelid. Nodules contained germinal centers by 2 WPH and plasma cells by 4 WPH. The epithelium associated with these nodules was flattened, had short, irregular microvilli, contained intraepithelial lymphocytes (IEL), and lacked goblet cells. High endothelial venules were located at the base of lymphoid nodules and contained lymphocytes within and below the cuboidal endothelium. In the upper eyelid, CALT was morphologically similar to lymphoid tissue in the lower eyelid, but nodules were smaller and more random, lacked an association with epithelial folds and fissures, and were clustered around the opening of the naso-lacrimal duct. By 12 WPH, CALT was characterized by the presence of basal germinal centers outlined by collagenous stroma, suprafollicular plasma cells, columnar epithelium with goblet cells, and fewer IEL. Based on these features, CALT in chickens has morphologic characteristics similar to other components of the mucosal immune system and therefore may have a role in mucosal immunity.

INTRODUCTION

Conjunctiva-associated lymphoid tissue (CALT), recently described in rabbits,[13,47,78] guinea pigs,[47] and turkeys,[71] has been proposed to function as a component of the mucosal immune system.[13,47,71,78] The mucosal immune system is important for immunologic protection along mucosal surfaces. Unique features of this system include antigen uptake through specialized epithelium[33,145,183] and transepithelial delivery of secretory immunoglobulin.[22,23,87] In mammals, gut-associated lymphoid tissue (GALT)[24,146] and bronchus-associated lymphoid tissue (BALT)[20,27,28,30] have received the most study; however, similar tissue has also been identified in salivary ducts,[126] endometrium,[122] and the nasopharynx.[127] Although GALT and BALT have been identified in birds,[10,16,27,82,118,149] the avian Harderian gland has also received considerable attention regarding its role in mucosal immunity.[5,14,82,149]

The chicken Harderian gland is a tubulo-acinar secretory gland which contains a significant plasma cell population by several weeks of age.[171,199] The infiltrating plasma cells are located in the glandular interstitium, have a bursa-dependent development,[4,179] and secrete IgA.[5] The retrobulbar location of the Harderian gland provides only limited access to the conjunctival mucosa. Secretions empty into the conjunctival space via a duct; subsequent drainage into the upper respiratory tract occurs through the naso-lacrimal duct.[14] Eyedrop delivery of antigen produces an increase in the numbers of plasma cells in the Harderian gland,[181] specific antibody appearance in its secretions,[41] and minor development of lymphoid follicles.[61] The site of antigen uptake that leads to plasma cell generation and localization in the Harderian gland is currently unknown.

Since the control of several economically important chicken diseases is currently attempted through eyedrop,

aerosol, and oral vaccination, mucosal immunity is likely important in the success of these nonparenteral vaccination strategies.[119,135] Many of these vaccines may induce protective immunity through contact with mucosal lymphoid tissues. Since vaccine delivery by eyedrop, aerosol, and oral exposure also provides conjunctival contact, lymphoid tissue within the conjunctiva may be important in antigen uptake. The purpose of this study was to describe anatomical features of chicken eyelids pertinent to CALT, characterize the gross, histologic, and ultrastructural features of CALT in chickens, and address the possible role of this tissue in mucosal immunity.

MATERIALS AND METHODS

One-day-old broiler chickens (n = 108) were obtained from a local hatchery (Hoover Hatchery, Rudd, Iowa) after debeaking and subcutaneous administration of a killed Marek's disease virus vaccine in the dorsal cervical region. Chickens were housed in a well-ventilated room in an indoor laboratory animal facility concurrently housing no other chickens. After 2 weeks in temperature-controlled brooders, chickens were moved to wire cages for 2 additional weeks, then placed on concrete flooring with sawdust litter for the remainder of the study. Free access to fresh water and feed was provided at all times.

At 1 day and 1, 2, 3, 4, 6, 8, 12, and 16 weeks post-hatching (WPH), 12 chickens were euthanatized by intravenous administration of sodium pentobarbital. Upper and lower eyelids were removed immediately with special care to keep the naso-lacrimal duct opening and conjunctiva intact. Upper and lower eyelids from each bird were maintained as a set, and a group of sets from 4 birds was fixed in either 3% acetic acid, 10% neutral buffered formalin, or 3% glutaraldehyde in 0.05 M Sorensen's phosphate buffer, pH 7.3.

For gross examination of CALT, eyelids fixed in acetic acid were prepared as previously described.[57] Briefly, eyelids were fixed for 24 hours in 3% acetic acid, stained in 0.5% aqueous methylene blue for 1 minute, and then washed in cold water for 2 to 3 hours to remove stain from non-lymphoid areas. Washing maximized contrast between lymphoid and non-lymphoid tissues. Eyelids were placed on glass and stained lymphoid tissue was visualized by transillumination with a dissecting microscope.

For histologic evaluation, cross sections of formalin-fixed eyelids were trimmed perpendicularly to the free lid margin, processed routinely by paraffin technique,

and sectioned at 4 μm. Eyelids were then stained with hematoxylin and eosin (HE), periodic acid-Schiff, or Masson's trichrome.

Eyelids fixed in glutaraldehyde were post-fixed in 1% osmium tetroxide, sequentially dehydrated in ethanols, and embedded in epoxy resin. Thin sections were cut with an ultramicrotome, stained with 2% methanolic uranyl acetate and Reynolds' lead citrate as previously described,[155] and examined with a Hitachi HS-9 transmission electron microscope.

RESULTS

All chickens remained healthy and free of clinical disease throughout the study period. By gross and histologic examination, CALT was not detected in the upper or lower eyelids of any 1-day-old chickens.

Eyelid structure in 1-day-old chickens

The lower eyelid conjunctiva in 1-day-old chickens had longitudinal epithelial folds and fissures running closely parallel to the conjunctival fornix (Fig. 1). Macroscopically, these folds and fissures extended from the nasal palpebral commissure toward the temporal palpebral commissure in the proximal part of the conjunctiva. In these areas, tall columnar epithelial cells and goblet cells covered a central fibrovascular connective tissue stroma (Fig. 2). This supporting stroma contained scattered subepithelial lymphocytes and prominent capillaries. Ultrastructurally, electron dense basal epithelial cells along a continuous basement membrane were located below more superficial, less electron dense columnar cells (Fig. 3). Epithelial cells had intercellular junctions, intermediate filaments, clear vesicles, and prominent apical microvilli. Capillaries appeared immediately below the basement membrane.

In contrast to the lower eyelid, the upper eyelid in one-day-old chickens had two major components: an outer feathered portion lined by a stratified squamous conjunctival epithelium, and an inner fibrous portion that originated from the conjunctival side of the outer portion (Fig. 4). This inner portion extended toward the eyeball and had a columnar epithelium that formed a series of small, regular ridges parallel to the free lid margin. The proximal folds and

fissures characteristic of the lower eyelid were lacking in the upper eyelid.

Lymphoid component of CALT

Lymphoid tissue was not found in the eyelids of 1-day-old chickens. However, in subsequent weeks, lower eyelid folds contained increasingly prominent lymphoid nodules (Fig. 5). At 1 WPH, isolated clusters of lymphocytes formed small subepithelial nodules randomly along the parallel folds in the lower eyelid proximal conjunctiva. By 2 WPH, larger lymphoid nodules obliterated adjacent fissures and formed confluent areas of lymphoid infiltration (Fig. 6). These large lymphoid nodules contained a mixed population of lymphocytes, lymphoblasts, and macrophages and greatly expanded individual proximal conjunctival folds. Germinal centers in these nodules were numerous, contained lymphoblasts in mitosis, and occurred in both superficial and deep CALT regions (Fig. 7). By 12 WPH, germinal centers were deeper than at earlier weeks and were isolated from surrounding lymphoid tissue by a prominent circumferential collagenous stroma. Occasional solitary plasma cells were subepithelial at 3 WPH; by 4 WPH, there were numerous small clusters of subepithelial plasma cells. At 16 WPH, plasma cells and mature lymphocytes populated suprafollicular areas but interfollicular areas contained a more heterogenous population of lymphocytes, lymphoblasts, and macrophages. Occasional intraepithelial plasma cells were between basal epithelial cells after 4 WPH. In some chickens after 6 WPH, diffuse intraepithelial lymphocytes (IEL), subepithelial lymphocytes, and epithelial goblet cell metaplasia were variably present along the normally lymphocyte-free pseudostratified conjunctiva from the extreme proximal edge of CALT to the conjunctival fornix.

In the upper eyelid conjunctiva, macroscopic CALT nodules were considerably fewer than in the lower eyelid and were characteristically clustered around the opening of the naso-lacrimal duct (Fig. 8). Histologically, several small lymphoid aggregates were below the columnar epithelial ridges of the inner fibrous flap at 2 WPH. Comparable nodules were not evident below the stratified epithelium of the outer feathered portion until 4 WPH. Nodules in both areas were more prominent at 6 WPH, contained occasional germinal centers, but remained solitary. At 12 WPH, nodules were confluent in some areas and diffuse subepithelial lymphoid infiltrates were occasionally seen. However, upper eyelid CALT never reached the degree of development seen in the lower eyelid. Goblet cell metaplasia was occasionally found in conjunctival epithelium of the upper eyelid.

Epithelium of CALT

In areas of CALT, a flattened epithelium covered lymphoid nodules and contained numerous IEL (Fig. 7). From 1 to 4 WPH in lower eyelids, epithelial cell flattening, lymphocyte infiltration, and lack of goblet cells occurred concurrently and progressively with subepithelial lymphoid nodule development. Generally, epithelial cells at the top of conjunctival folds were flatter than cells along adjacent fissures. Extreme infiltration by IEL, concurrent with maximal development of CALT at 3 to 6 WPH, made histologic delineation of a distinct epithelium difficult. Ultrastructurally, epithelial distortion and disruption was evident as IEL and plasma cells infiltrated the epithelium across a discontinuous basement membrane (Fig. 9). Flattened epithelial cells had short, irregular microvilli, distinct linear bundles of intermediate filaments, apical vesicles, and prominent intercellular junctions (Fig. 10). Basal epithelial

cells were more electron dense than superficial epithelial cells. The epithelial basement membrane was frequently interrupted by cytoplasmic processes of lymphocytes. Although initially solitary, IEL were in small clusters by 2 WPH. IEL interdigitated with disrupted and distorted processes of flattened epithelium, but lacked intercellular junctions (Fig. 10). By 12 WPH, epithelial cells in some segments were again taller, basal cells had a more distinct basement membrane, and IEL were less numerous. In addition, goblet cells previously absent within the flattened epithelium were present once again along conjunctival folds.

The developmental features of CALT epithelium within the upper eyelid were similar to those within the lower eyelid. However, since upper eyelid CALT was not as prominent, epithelial flattening and IEL infiltration were not as extensive.

Vessels of CALT

Characteristic blood vessels observed in CALT by transmission electron microscopy included subepithelial capillaries and high endothelial venules (HEV). In 1-day-old chickens, capillaries examined by transmission electron microscopy immediately below the epithelium in proximal lower eyelid folds had long, thin endothelial processes (Fig. 11). These capillaries also frequently contained intraluminal lymphocytes. As CALT developed, the endothelial processes of these capillaries became more attenuated, with shorter processes persisting in chickens after 6 WPH. In addition to these subepithelial capillaries, HEV developed concurrently with CALT (Fig. 12). The location of these venules was restricted to the base of lymphoid nodules. HEV frequently contained lymphocytes that formed close membrane contacts with endothelium by cytoplasmic projections. Lymphocytes were

located between and immediately below adjacent endothelial cells, crossed the HEV wall, and formed dense clusters around HEV. In contrast, lymphocytes were not seen in any of these locations in the superficial subepithelial capillaries described above. HEV of similar structure and location were identified histologically below lymphoid nodules in the upper eyelid.

Fig. 1: Cross section of the lower eyelid, 1-day-old chicken. F = proximal conjunctival fornix, M = distal free lid margin. Proximal conjunctival folds and fissures where lymphoid tissue develops (arrow). HE. Bar = 1 mm

Fig. 2: Proximal conjunctival folds and fissures, 1-day-old chicken (area indicated by arrow in Fig. 1). Tall columnar epithelial cells and goblet cells along a fibrovascular stroma. HE. Bar = 50 µm

Fig. 3: Electron photomicrograph of epithelium in proximal folds and fissures, 1-day-old chicken (area indicated by arrow in Fig. 1). Electron dense basal cells along a continuous basement membrane (arrows). Bar = 5 µm


M
F
1

Fig. 4: Cross section of the upper eyelid, 1-day-old chicken. O = outer feathered portion, I = inner fibrous portion. Regular epithelial ridges along inner portion (arrow). HE. Bar = 1 mm

Fig. 5: Gross photogragh of the lower eyelid, 3-week-old chicken. Note linear association of dark lymphoid nodules with faint proximal conjunctival epithelial folds and fissures. Acetic acid fixation, aqueous methylene blue. Bar = 3 mm

Fig. 6: Proximal conjunctival folds and fissures, lower eyelid, 2-week-old chicken. Note expansion of conjunctival folds by lymphoid nodules; compare to Fig. 2. HE. Bar = 100 μm

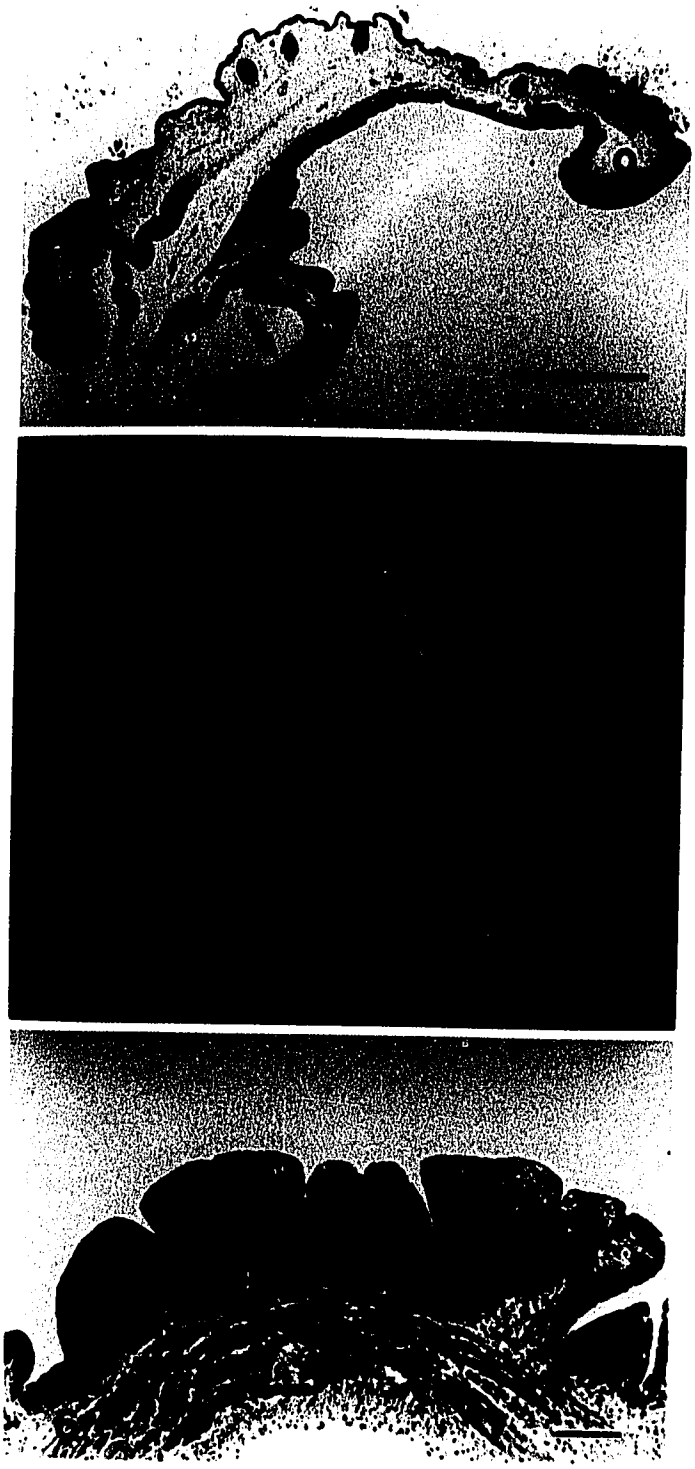


Fig. 7: Detail of conjunctival fold from Fig. 6. GC = germinal center. Note superficial flattened epithelium and obscure basement membrane (arrows). Compare to Fig. 2. HE. Bar = 25 μm

Fig. 8: Gross photograph of the upper eyelid, 3-week-old chicken. N = opening of the naso-lacrimal duct. Note clustering of dark lymphoid nodules around duct opening. Acetic acid fixation, aqueous methylene blue. Bar = 3 mm

Fig. 9: Electron photomicrograph of lymphoid nodule, 4-week-old chicken. L = intraepithelial lymphocyte, PC = plasma cell. Note lymphocyte across a discontinous basement membrane (arrow). Bar = 3 μm

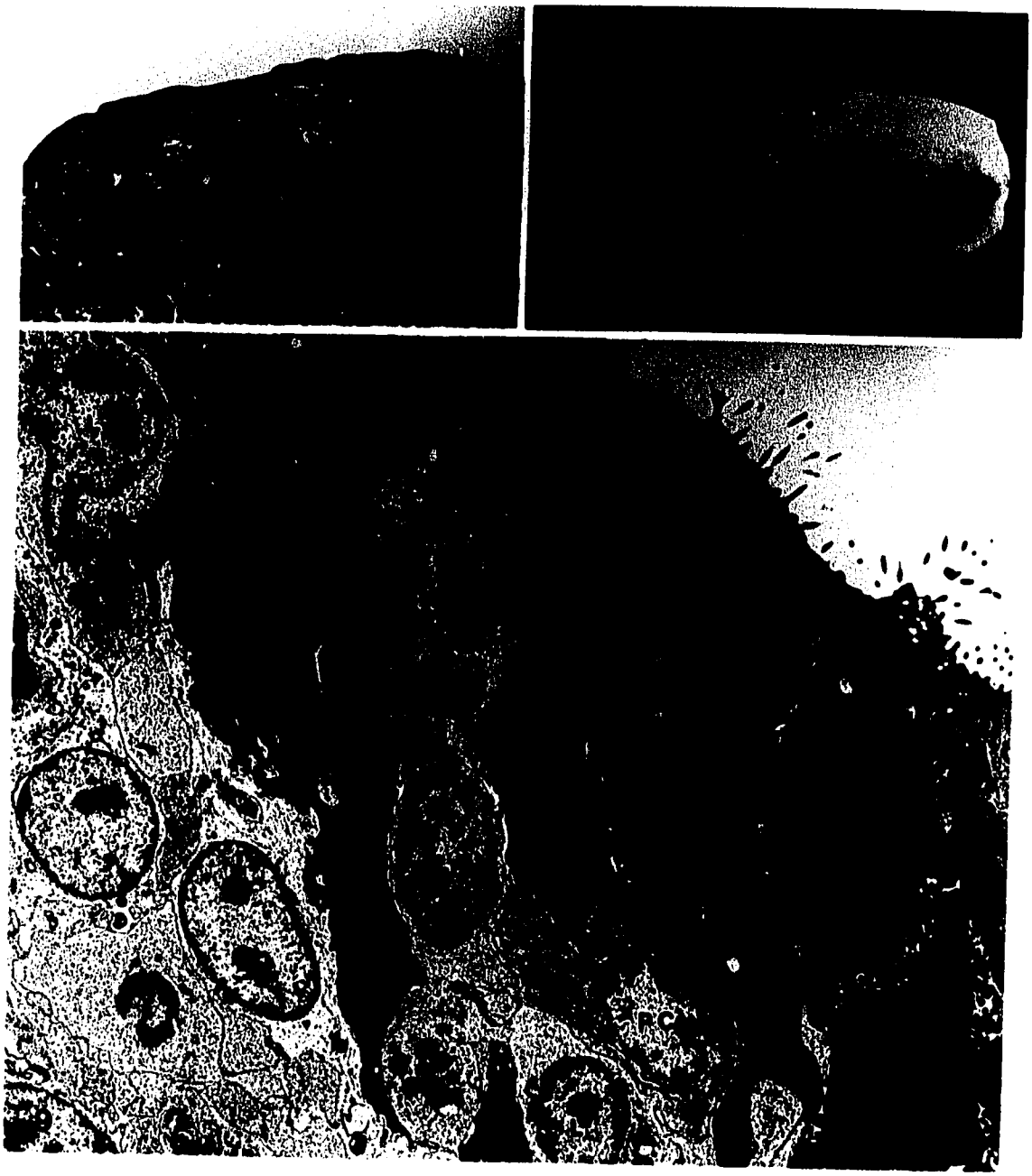


Fig. 10: Electron photomicrograph of epithelium over a lymphoid nodule, 2-week-old chicken. I = intermediate filaments, L = intraepithelial lymphocyte. Note irregular microvilli, prominent intercellular junctions between epithelial cells (arrows), and apical vesicles (arrowheads). Bar = 1 μm

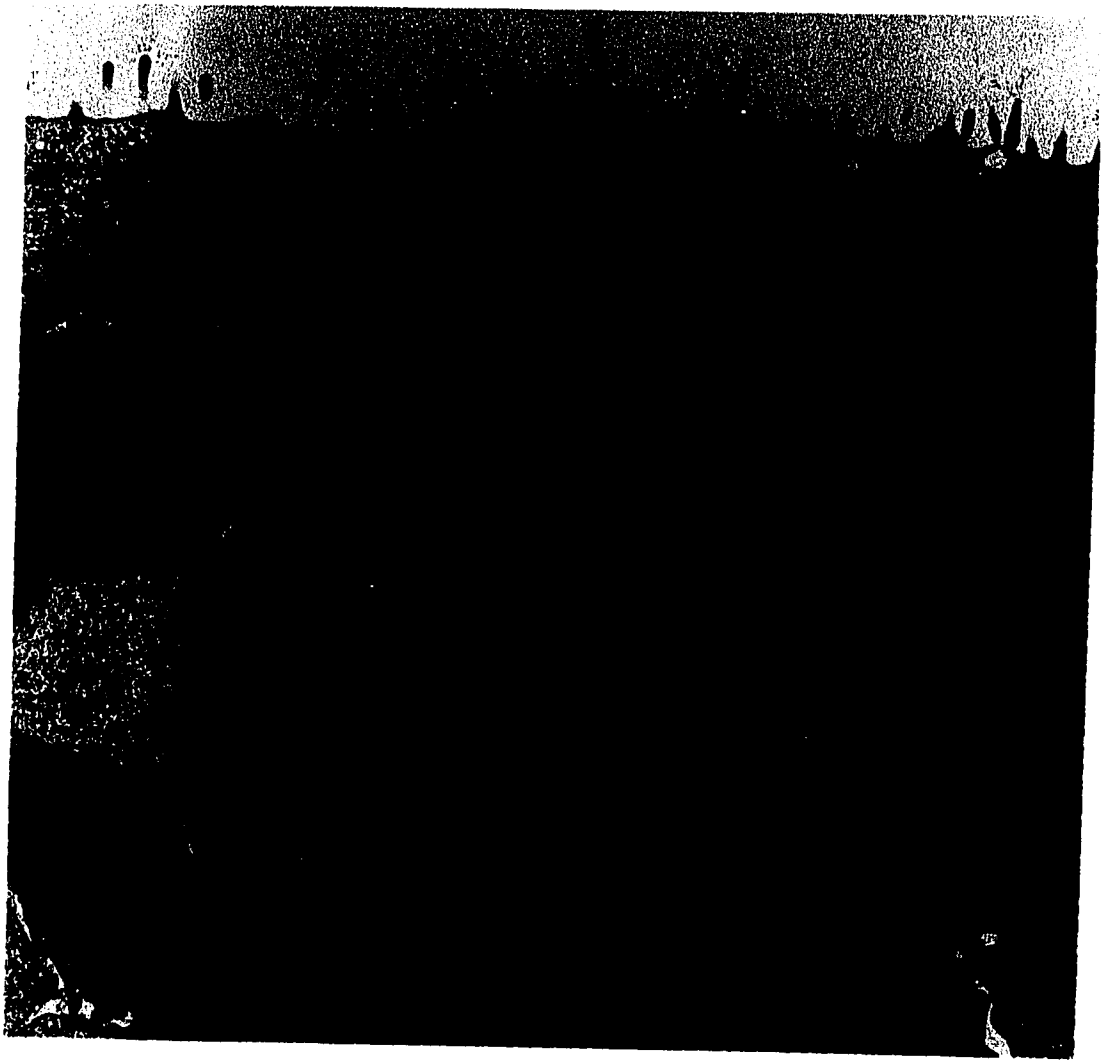
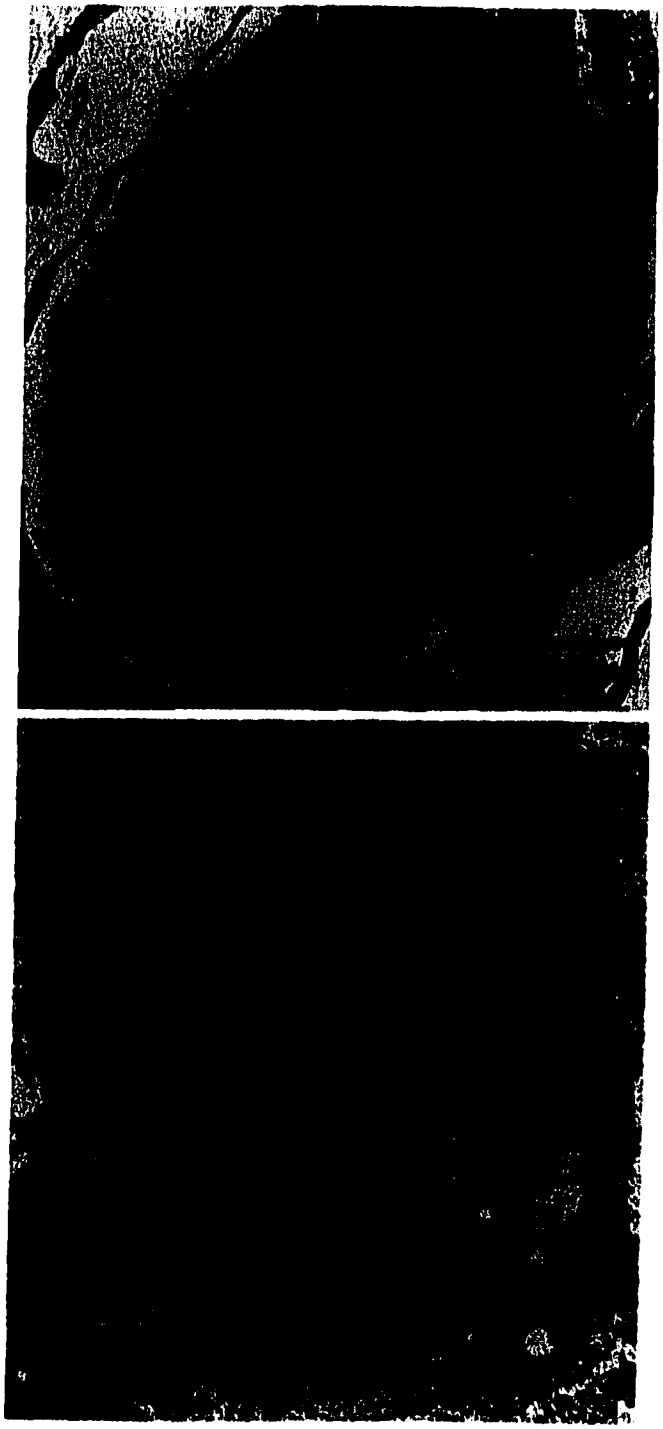


Fig. 11: Electron photomicrograph of a subepithelial capillary, 1-day-old chicken. E = endothelial cell, L = lymphocyte. Note long endothelial processes contacting lymphocyte. Bar = 2 μm

Fig. 12: Electron photomicrograph of a high endothelial venule, 4-week-old chicken. E = endothelial cell, L = lymphocyte, R = red blood cell in venule lumen. Note thickness of endothelium compared to Fig. 11. Bar = 3 μm



DISCUSSION

CALT in chickens has morphologic characteristics which are similar to other mucosa-associated lymphoid tissues. GALT and BALT both consist of heterogenous nodules of lymphocytes and macrophages within a loose stroma, an overlying attenuated epithelium composed of cells with short microvilli, epithelial infiltration by lymphocytes, and relatively few goblet cells.[16,24,30] These tissues typically contain specialized M-cells which allow the uptake of antigen across the epithelial surface.[30,128,202] In addition to characteristic lymphoid nodules and epithelium, mucosal lymphoid tissues contain HEV which are specialized for regulating the migration of lymphocytes into specific lymphoid tissues.[193,204] Since CALT in chickens has many of the above characteristics, it likely has a function in mucosal immunity.

The location and general structure of CALT in chickens are similar to CALT in turkeys.[71] In both species, CALT is present most prominently in longitudinal folds and fissures of the lower eyelid, appears shortly after hatching, and has similar epithelial and vascular histomorphologic features.[71] Although CALT has not been described in turkeys over 33 days of age,[71] older turkeys also have features of CALT similar to the older chickens in the present report (A. S. Fix, personal observation).

Differences exist between CALT in chickens and in turkeys. The most apparent differences include more prominent lymphoid nodules in the chicken upper eyelid and the clustering of these nodules around the opening of the naso-lacrimal duct. Since this duct drains ocular secretions, antigens and microorganisms in these secretions would make contact with lymphoid tissue around the duct opening. Mucosal lymphoid tissue in this location may optimize the chance for antigen contact and uptake. Other characteristics of chicken

CALT not found in turkey CALT[71] include diffuse lymphoid infiltrates along the proximal conjunctiva near the lower eyelid fornix, a prominent plasma cell component, epithelial goblet cell metaplasia in both upper and lower eyelids, and superficial subepithelial capillaries with long endothelial processes. In chickens, CALT development was prominent at an earlier age than in turkeys.[71]

CALT in chickens is less similar to CALT in rabbits and in guinea pigs than to CALT in turkeys. In contrast to the relatively early development of CALT in chickens, CALT in rabbits does not appear until after 4 weeks of age.[13] Additionally, chicken CALT has prominent germinal centers, an attenuated epithelium, numerous plasma cells, and HEV. None of these features are described in CALT of rabbits or of guinea pigs.[13,47] Rabbit CALT has prominent lymphocyte-packed lymphatic channels not seen in the chickens of the present study.[13] However, the greater number of upper eyelid conjunctival lymphoid nodules in our chickens more closely resembles the distribution of CALT in rabbits than in turkeys.[13,71]

The characteristic morphologic features of CALT development in chickens over the 16 weeks of this study may suggest a change in function. After hatching, conjunctival lymphoid infiltration and epithelial modification occurred gradually until development peaked at 3 to 6 WPH. However, after 12 WPH, the epithelium was once again columnar with goblet cells, had a more prominent basement membrane, and contained less numerous IEL. In addition, CALT germinal centers were more basal in nodules and were surrounded by a collagenous circumferential stroma. Also concurrent with CALT development was the progressive increase in plasma cells within and below the epithelium. These morphologic differences in CALT between younger and older chickens suggest the early ability of CALT to sample and react to antigens in

the conjunctival space, with a later emphasis on the delivery of antibody to the conjunctival surface from superficial plasma cells.

Considerable evidence exists supporting a role for the chicken Harderian gland in local paraocular immunity. Maximal infiltration by plasma cells into this gland occurs by about 6 weeks of age.[199] By immunofluorescence, most of these cells stain for IgM in chickens under 4 weeks of age, IgG- and IgA-positive cells occur with equal frequency until 11 weeks of age, and IgA-positive cells predominate in older chickens.[5] Several reports have described lymphoid follicle formation and specific antibodies in Harderian gland secretions after eyedrop delivery of antigens such as bovine serum albumin, sheep red blood cells, Newcastle disease virus, and infectious bronchitis virus.[41,61,153,181] However, detailed understanding of the immune response to these antigens and Harderian gland infiltration with plasma cells is currently lacking.

CALT in chickens may serve as a site for antigen uptake and processing, followed by localization of antibody-producing plasma cells to the Harderian gland. A similar role has recently been proposed for CALT in rabbits, guinea pigs, and turkeys.[13,47,71] If this is also true for chickens, some of the increased appearance of plasma cells and specific antibody in the Harderian gland after eyedrop delivery of antigen may be explained.[153,181] Based on this hypothesis, antigen introduced along the conjunctival epithelium would contact the subepithelial lymphoid population after crossing the specialized epithelium in CALT by a currently unknown mechanism. Subsequent to the generation of an immune response, committed B-cells would mature into antibody-producing plasma cells, localize within the Harderian gland, and deliver antibody into Harderian secretions. CALT would then provide the affector function in local paraocular

immunity, with the effector function provided by the plasma cell-rich Harderian gland.

This study has characterized structural features of CALT in chickens and documented a similarity to many mucosal lymphoid tissues in other species. CALT therefore has a probable role in mucosal immunity. Based on previous documentation of plasma cells in and antibody production by the chicken Harderian gland, CALT may serve as a site of antigen uptake within the conjunctiva. If this proves to be true, a better understanding of the interaction between nonparenteral vaccines and CALT may enhance the control of economically significant respiratory diseases in chickens.

PART III. PARTICLE UPTAKE BY CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN TURKEYS

PARTICLE UPTAKE BY CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN TURKEYS

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ABSTRACT

Uptake of tracer particles was assessed in lower eyelid conjunctiva-associated lymphoid tissue (CALT) of 3-week-old turkeys. Tracer particle suspensions (carbon, iron oxide, or three sizes of latex beads - 0.81 μm, 1.7 μm, and 2.9 μm) were placed into the experimentally sealed conjunctival space. After 5, 15, or 30 minutes, eyelids were removed and CALT was examined by light microscopy. Uptake was confirmed for all tracers and occurred within the lymphoepithelium of CALT. The uptake of latex beads was not as frequent as for carbon and iron. Tracers were increasingly evident in lymphoepithelium and subepithelial macrophage clusters as contact time increased. These findings provide additional evidence that CALT is capable of antigen uptake and may play a role in paraocular and upper respiratory immunity in turkeys.

INTRODUCTION

Conjunctiva-associated lymphoid tissue (CALT) is a recently described entity in turkeys that has many of the structural features of mucosa-associated lymphoid tissues.[71] CALT is associated with distinct folds and fissures in the proximal region of the lower-eyelid conjunctiva. Although not present at hatching, CALT develops into prominent lymphoid nodules by 3 weeks of age. Morphologic features include dense lymphoid infiltrates below a flattened lymphoepithelium, intraepithelial lymphocytes, lack of goblet cells, and high endothelial venules.[71] CALT in turkeys resembles that described in rabbits and guinea pigs[13,47,78] and undergoes hyperplasia in response to local Bordetella avium infection.[71] The location and structural similarity of CALT with other mucosal lymphoid tissues, including gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT),[24] has led to the proposal that CALT is a functional component of the mucosal immune system.[13,47,71,78]

Mucosal lymphoid tissues are important in the defense of mucosal surfaces through the uptake of microbial antigens and the generation of a protective antibody response, primarily in the form of secretory IgA.[22] These tissues contain a specialized epithelium that is adapted for the transepithelial uptake of antigens and microorganisms.[202] A unique cell type, the M-cell, performs this function in the human gastrointestinal tract.[144] Similar epithelial types are described in other tissues and are referred to as a follicle-associated epithelium or lymphoepithelium.[202] Many studies have documented the uptake of tracer particles (carbon, latex beads, horseradish peroxidase) by the lymphoepithelium of mucosal lymphoid tissues.[92,129,140,183,198] Additional studies have demonstrated that the uptake of viruses,[203] bacteria,[145] and protozoa[141] also occurs.

Vaccination via mucosal exposure is critical for protective immunity against many infectious diseases.[119] The avian Harderian gland, a tubulo-alveolar gland containing plasma cells and secretory IgA, has been recognized as having a major role in local ocular immunity.[14,180] However, this gland lacks many structural features of mucosal lymphoid tissues, including a distinct lymphoepithelium, prominent intraepithelial lymphocytes, and high endothelial venules. In addition, although carbon tracer enters the duct of the Harderian gland after experimental eyedrop delivery, uptake across the epithelium was not detected.[42] The present study was designed to determine if tracer particle uptake occurs in CALT of turkeys and if uptake is influenced by tracer type and time of exposure.

MATERIALS AND METHODS

Fifty 1-day-old Nicholas strain broad-breasted white turkeys were obtained from a commercial hatchery (Midwest Turkey Hatchery, Dike, Iowa) and housed in an isolated room away from other animals within a well-ventilated indoor laboratory animal facility. Turkeys were kept in temperature-controlled brooders and were given starter ration and water ad libitum. No vaccinations were administered at the hatchery or during the experiment.

Tracer particle suspensions (carbon, iron, latex beads) were prepared to assess uptake by conjunctiva-associated lymphoid tissue (CALT) in the lower eyelid. For carbon, 1 ml of black India Ink (Faber-Castell Corp., Newark, New Jersey) was diluted in 20 ml phosphate-buffered saline (PBS) and centrifuged at 5000 x g for 30 minutes. After the supernatant was discarded, the pellet was resuspended in 10 ml PBS. For iron, 100 mg of black iron oxide particles (0.5 μm diameter, Polysciences, Inc., Warrington, Pennsylvania) was suspended in 10 ml PBS. For latex beads, three different diameters were selected (0.8 μm, 1.7 μm, and 2.9 μm). One ml of stock suspension from each size (Polystyrene Microspheres, 2.5% solids-latex, Polysciences, Inc.) was centrifuged at 3000 x g for 10 minutes, and, after the supernatant was discarded, the pellet was resuspended in 10 ml PBS. All tracer suspensions were warmed to approximately 38 C before use.

An experimental technique was developed to control tracer particle suspension contact with CALT. At 3 weeks of age, turkeys were anesthetized with sodium pentobarbital, and eyelid margins were sealed bilaterally with adhesive (Duro Superglue-5, Woodhill Permatex, Cleveland, Ohio). Each naso-lacrimal duct was ligated with a single subcutaneous suture anterior to the nasal palpebral commissure to seal the conjunctival space. Preliminary light microscopic evaluation

of eyelids prepared by this technique ensured that the conjunctival space was closed and CALT was not morphologically damaged. Tracer suspensions were injected by syringe with a 22-gauge needle through the upper eyelid into the sealed conjunctival space of the right eye. Sufficient tracer suspension was injected to ensure adequate contact with CALT (1 ml). Each tracer suspension was injected into the conjunctival space of nine turkeys. For negative controls, PBS without tracer was injected into the left sealed conjunctival space of each bird. For positive controls and to assess the microscopic appearance of tracer material in liver and spleen, five additional turkeys received 0.5 ml of each tracer suspension intravenously.

At 5, 15, and 30 minutes postinjection (PI), three turkeys from each tracer group were sacrificed with an intravenous overdose of sodium pentobarbital. Lower eyelids were removed and fixed in 10% neutral buffered formalin. After 24 hours, the middle of each eyelid was trimmed in cross-section perpendicular to the eyelid margin. Positive control turkeys receiving intravenous tracer were sacrificed in a similar manner at 5 minutes PI. Liver and spleen were harvested immediately and fixed in 10% formalin. All tissues exposed to carbon and iron were processed by routine paraffin technique, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) for light microscopy or fast green (FG) for photomicrography.[170] Tissues exposed to latex beads were embedded in plastic (glycol methacrylate, GMA Kit, Polaron, Cambridge, Massachusetts), sectioned at 2 μm, and stained with 0.1% aqueous toluidine blue (TB) as described.[151]

A minimum of 10 sections from each block were examined by light microscopy. Observations were recorded with particular attention to tracer location within CALT at the specific intervals PI. Microscopic assessment of uptake was restricted

to the CALT lymphoepithelium and nonlymphoid epithelium in proximal conjunctival folds and fissures of the lower eyelid.

RESULTS

All tracer particles had a characteristic histologic appearance in positive control tissues (liver and spleen). In liver, tracers were located in Kupffer cells along hepatic sinusoids. Similarly, splenic tracer particles were confined to phagocytic cells associated with sheathed arteries (Schweiger-Seidel sheaths). Carbon particles were numerous, appeared as uniform 1 to 2 μm light-brown granules, and occasionally formed large clumps filling cell cytoplasm (Fig. 1). Iron particles were black and irregular, with a tendency to clump into aggregates of 5 μm diameter or more. Fast green (FG) stain provided better contrast for tracer visualization than hematoxylin and eosin (HE). However, FG provided poor cellular detail of the tissue. In plastic sections stained with toluidine blue (TB), latex beads appeared as small, round to oval, clear cytoplasmic spaces. By adjusting the microscope condenser to enhance contrast, latex beads were distinctly refractile and easily identified. Generally, the number of beads within a given cell was inversely proportional to the size of the bead. Cellular detail in TB-stained plastic sections was superior to those embedded in paraffin. No tracer material was observed in any eyelid section from saline-injected negative controls.

Carbon, iron oxide, and latex beads were located primarily within or below the conjunctiva-associated lymphoid tissue (CALT) lymphoepithelium, although occasionally these tracers were also seen in the more typical nonlymphoid epithelium of the conjunctiva. The appearance of carbon and iron oxide particles in CALT was similar to positive control liver and spleen sections, although some larger aggregates of iron oxide (greater than 6.0 μm) were displaced artifactually by sectioning. This was verified by evaluation of additional serial sections. By 30 minutes post-injection (PI), increased

numbers of heterophils were observed near the conjunctival epithelium in a few sections.

At 5 minutes PI, carbon and iron oxide particles were within the CALT lymphoepithelium in approximately one-half of the sections examined. These tracers appeared as small (2 to 4 μm) granules (Fig. 2). In a few areas, carbon and iron oxide particles were concentrated in small clumps (5 to 8 μm) immediately below the lymphoepithelium. By 15 minutes PI, larger and more distinct clumps (10 to 15 μm) were typical (Fig. 3). These areas were located further below the lymphoepithelium than at 5 minutes and suggested localization in subepithelial macrophages. At 30 minutes PI, tracer particles were more dispersed within CALT and were in deeper sites than at previous times (Fig. 4). The distinct clumping observed earlier was not as evident. Occasional tracer-laden phagocytic cells (macrophages and heterophils) were seen above the lymphoepithelial surface or free in the conjunctival space. Small, thin cytoplasmic strands extended between these cells and the underlying lymphoepithelium.

Localization of all three sizes of latex beads in the CALT lymphoepithelium was less common than for carbon and iron. Generally, 0.81 μm beads were located superficially, near the top of lymphoepithelial folds. However, the 1.7 μm and 2.9 μm beads were located in the lymphoepithelium near the base of conjunctival fissures between adjacent folds. The small 0.81 μm latex beads were seen in dense clusters below the superficial lymphoepithelium only at 30 minutes PI (Fig. 5), although occasional solitary beads were also observed. A solitary 1.7 μm bead was seen in the lymphoepithelium of one section at 5 minutes PI. Other sections contained solitary and loose clusters of beads within and below the lymphoepithelium at 30 minutes (Fig. 6). The 2.9 μm beads were solitary and within lymphoepithelial cells at 5 minutes PI. At 30 minutes PI, several of these large beads were also

in loose clusters below the lymphoepithelium (Fig. 7). The location of the 1.7 μm and 2.9 μm beads in the conjunctival fissure lymphoepithelium was in contrast to the superficial lymphoepithelial location of carbon, iron oxide, and 0.81 μm beads.

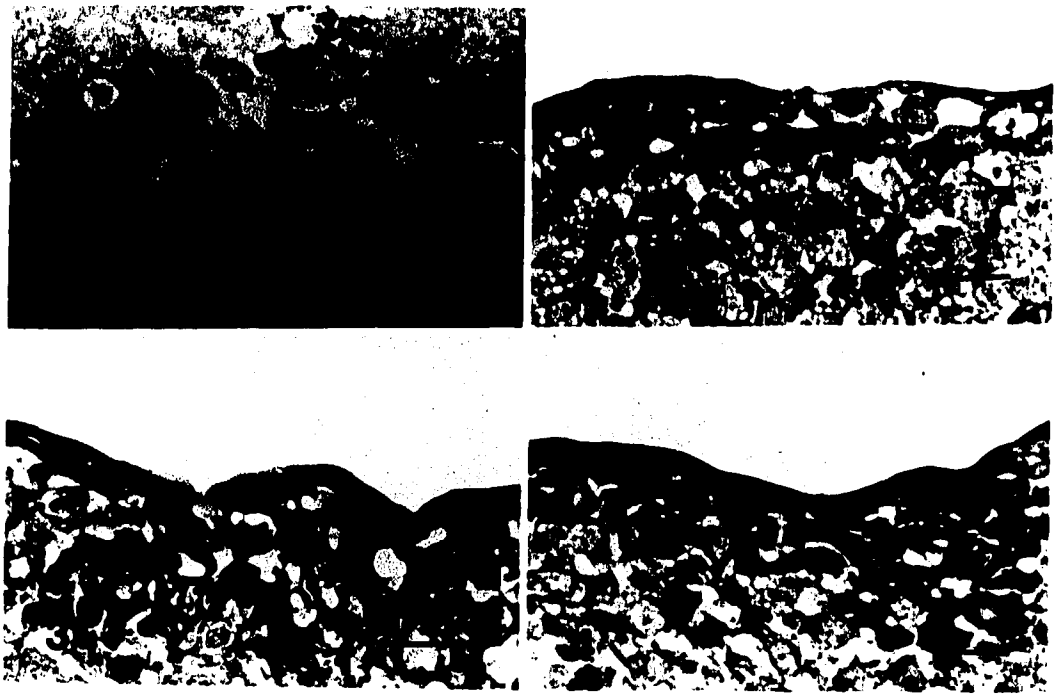
Figures 1 through 4. Uptake of carbon and iron oxide in 3-week-old turkeys

Fig. 1: Liver, positive control, intravenous carbon. Note clustering of carbon in Kupffer cells lining sinusoidal endothelium. FG. Bar = 10 μm

Fig. 2: Conjunctiva-associated lymphoid tissue, top of lymphoepithelial fold, carbon, 5 minutes PI. Tracer is primarily intraepithelial (arrow). FG. Bar = 10 μm

Fig. 3: Conjunctiva-associated lymphoid tissue, top of lymphoepithelial fold, carbon, 15 minutes PI. Tracer is clustered in subepithelial macrophages (arrow). FG. Bar = 10 μm

Fig. 4: Conjunctiva-associated lymphoid tissue, top of lymphoepithelial fold, iron oxide, 30 minutes PI. Tracer is deeper in lymphoid tissue and more dispersed than at earlier times (arrow). FG. Bar = 10 μm

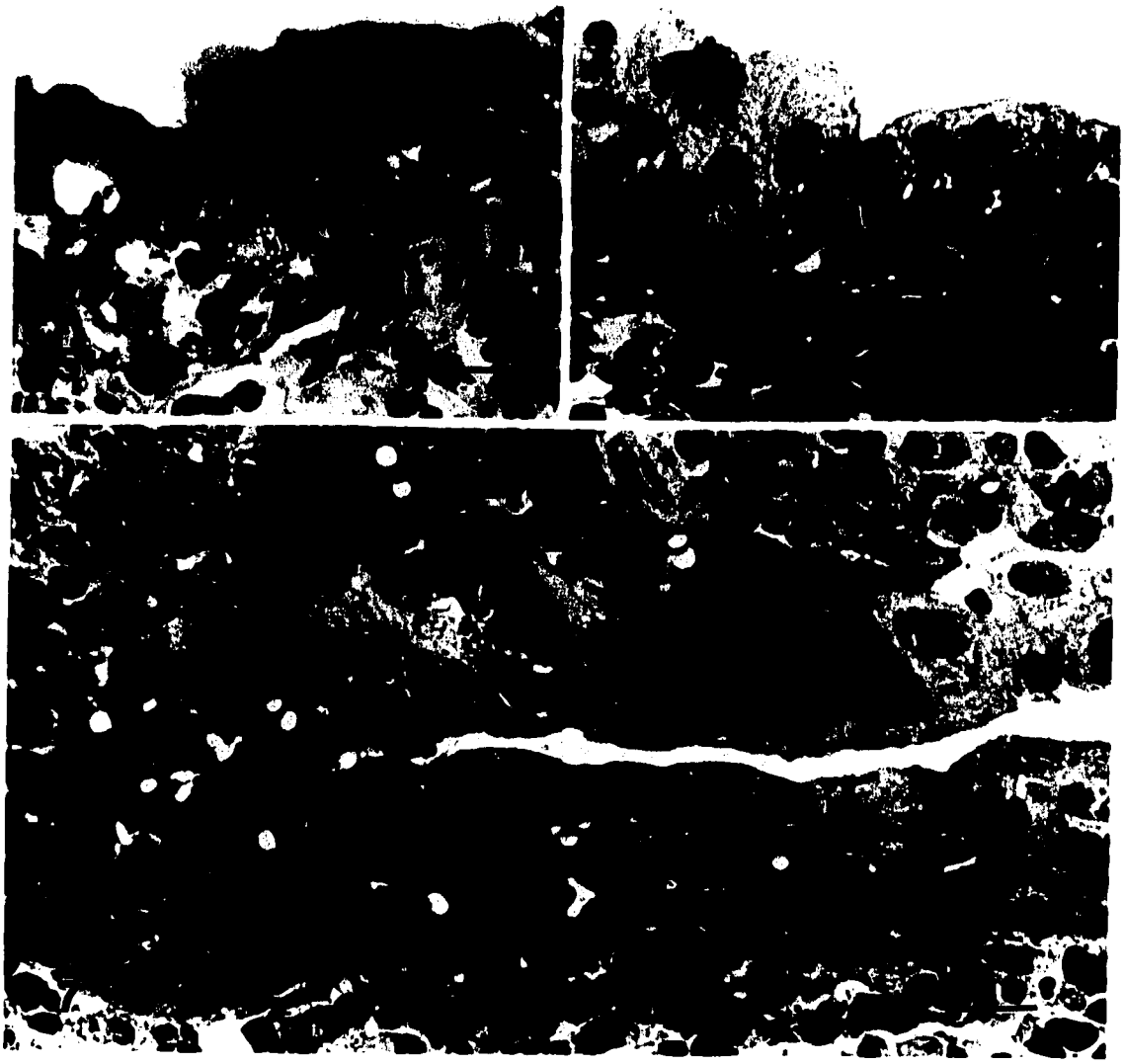


Figures 5 through 7. Uptake of latex beads in 3-week-old turkeys

Fig. 5: Conjunctiva-associated lymphoid tissue, top of lymphoepithelial fold, 0.81 μm latex beads, 30 minutes PI. Note clustering of numerous beads in a subepithelial macrophage (arrow). Solitary beads are also present. TB. Bar = 10 μm

Fig. 6: Conjunctiva-associated lymphoid tissue, lateral fissure, 1.7 μm latex beads, 30 minutes PI. Intraepithelial and subepithelial beads are solitary and in loose clusters (arrow). TB. Bar = 10 μm

Fig. 7: Conjunctiva-associated lymphoid tissue, lateral fissure, 2.9 μm latex beads, 30 minutes PI. Beads are primarily solitary (arrows) and in intraepithelial and subepithelial sites. TB. Bar = 10 μm



DISCUSSION

The experimental tracer particle studies evaluated in the present report support our earlier proposal that conjunctiva-associated lymphoid tissue (CALT) in turkeys has a role in mucosal immunity.[71] We have shown that the location of carbon, iron oxide, and latex bead tracers is restricted to the lymphoepithelium of CALT. Tracer localization within the lymphoepithelium of a mucosal lymphoid tissue is consistent with uptake of that tracer and structural modifications within CALT probably correlate with this function. The overall location in proximal conjunctival folds, the characteristic flattened lymphoepithelium, and intraepithelial lymphocytes all may facilitate contact between material in the conjunctival space and lymphoid cells. Tracer uptake has been documented in other mucosal immune system tissues, including gut-associated lymphoid tissue (GALT)[92,107,198] and bronchus-associated lymphoid tissue (BALT).[183,192] Interestingly, although the structure of CALT in rabbits and guinea pigs resembles CALT in turkeys,[13,47,71,78] tracer uptake is apparently not selective for the lymphoepithelial cells of guinea pig CALT.[178] In that study, experimental conjunctival delivery of horseradish peroxidase produced uptake in both lymphoid and nonlymphoid epithelial areas. This is in direct conflict with the present study, in which uptake was restricted to the lymphoepithelium of CALT.

Differences in the frequency and site of uptake in our study are likely a function of tracer size and composition. Trapping between conjunctival folds may have facilitated contact between the larger beads and the epithelium. The size of the larger beads may have limited phagocytosis of multiple beads by one macrophage. Because surface adsorption is apparently a prerequisite for uptake,[183] latex may not produce the same surface interaction between particle and epithelial

cell that occurs with carbon and iron. Uptake is also apparently influenced by the relative activity of nearby lymphoid tissue,[164,183] surface charge,[125] and tracer concentration.[106]

The precise cellular mechanism of uptake could not be determined with the histological observations in the present study. Although tracers appeared to be intraepithelial, the abundant intraepithelial lymphocyte population in CALT made the determination of exact cellular location difficult. An "intraepithelial" location was not necessarily synonymous with actual localization in the cytoplasm of an epithelial cell. Tracer particles could be in epithelial cell cytoplasmic vacuoles, within the processes of intraepithelial phagocytic cells, or between adjacent epithelial cells. Endocytosis and vesicle transport are the most common way that particles are taken up by GALT,[140] BALT,[183] and the bursa of Fabricius.[36] Transepithelial endocytosis could have occurred in our turkeys, since several of the larger beads appeared to be directly in epithelial cell cytoplasm. However, localization is also possible within macrophage pseudopods extended between epithelial cells, as described in Giardia muris uptake in mouse Peyer's patch epithelium.[141] Localization between adjacent epithelial cells would require disruption of epithelial cell tight junctions. This is less likely but has been observed with surgical manipulation in the guinea pig small intestine.[159] It is also possible that different tracers used in the present study were taken up by different mechanisms. Ultrastructural studies are required to definitively determine how tracer particles cross the lymphoepithelium of CALT in turkeys.

The detection of tracer-laden macrophages deeper in CALT after longer exposure time suggests active movement and migration of these cells within tissue. An interesting finding in the present study was the presence of tracer-laden

phagocytic cells on the lymphoepithelial surface and within the conjunctival space. Similar findings were reported when Vibrio cholerae was used to detect microbial uptake in rabbit Peyer's patches.[145] The movement and migration of phagocytic cells after uptake may have significance for antigen presentation and the generation of an immune response.

The experimental procedure developed in the present study was designed to maximize the opportunity for tracer uptake to occur, not to simulate conditions likely to be encountered in the field. The confirmation and characterization of uptake in CALT was our primary objective before attempting more applied experimentation. Admittedly, several aspects of the procedure, including anesthesia, surgical trauma, tracer delivery method, and the restriction of normal ocular movements, may have influenced the outcome. However, we believe that the uptake of biological materials via CALT is quite possible and may occur during natural infection of paraocular tissues and administration of eyedrop and aerosolized vaccines.

The uptake of tracer particles by CALT in the present study provides additional evidence that CALT is a component of the mucosal immune system and strengthens the specific role proposed for CALT in mucosal immunity of turkeys.[71] In the turkey Harderian gland, secretory IgA and plasma cells are present,[65,169,180] and the importance of this gland in avian paraocular immunity is recognized.[14,199] In chickens, conjunctival delivery of antigen produces an increase in Harderian gland plasma cells and specific antibody in ocular secretions.[61,153,181] We suggest that under field conditions, uptake of environmental microorganisms and vaccines through CALT results in the development of an immune response. This uptake may be responsible for a portion of the antibody production by the Harderian gland that contributes to protective immunity. The improved development of vaccines and

vaccination protocols specifically targeting CALT may enhance our attempt to control infectious disease in turkeys.

PART IV. QUANTIFICATION OF PARTICLE UPTAKE BY CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN CHICKENS

QUANTIFICATION OF PARTICLE UPTAKE BY CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE (CALT) IN CHICKENS

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ABSTRACT

Tracer particle uptake by conjunctiva-associated lymphoid tissue (CALT) was quantified in the lower eyelids of 1, 3, 5, and 7-week-old broiler chickens. CALT was measured histologically by computerized image analysis in all birds and in additional 1-day-old and 16-week-old chickens not subjected to uptake assessment. Suspensions of carbon or iron oxide were placed in contact with CALT for 5, 15, or 30 minutes (contact time). After eyelid removal, tracer uptake was scored by light microscopy, CALT was measured, and a mathematically-derived uptake index was evaluated statistically. At each age examined, computer-generated measurements showed a significant increase in the proportion of CALT lymphoepithelium within proximal eyelids. Tracer uptake was confined to CALT lymphoepithelium at all ages and at all contact times. Uptake increased significantly between 3 and 5 weeks of age and between 5 and 15 minutes of tracer contact. Based on these uptake data for CALT in chickens, a minimal age-specific maturity is suggested which may influence function in mucosal immunity.

INTRODUCTION

Conjunctiva-associated lymphoid tissue (CALT) is located primarily in the lower eyelid in turkeys,[71] chickens,[72] rabbits,[13,78] and guinea pigs.[47] In chickens and turkeys, CALT develops after hatching in association with distinct folds and fissures in the proximal conjunctiva.[71,72] As CALT develops, these conjunctival folds become progressively expanded by lymphoid nodules and develop a flattened epithelium containing lymphocytes and lacking goblet cells. The epithelium associated with these lymphoid follicles resembles specialized cells important for antigen uptake in other mucosal lymphoid tissues and is termed a follicle-associated epithelium or lymphoepithelium (LE).[202] During the post-hatching period, LE appears concurrently with the development of lymphoid nodules in proximal conjunctival folds and the original nonlymphoid epithelium (nonLE) gradually diminishes. CALT in chickens and turkeys[71,72] morphologically resembles other mucosal lymphoid tissues[16,24,70] which are important for the immunologic protection of mucosal surfaces.[22]

Tracer particle uptake is associated with mucosal lymphoid tissue LE and has been demonstrated experimentally using a wide variety of materials.[140,183,202] However, limited work has been done with CALT. The uptake of horseradish peroxidase has been documented in guinea pig conjunctiva, but no selectivity for CALT LE was found.[178] In contrast, we recently characterized the selective uptake of carbon, iron oxide, and latex beads by CALT LE in 3-week-old turkeys.[73] In our study, tracers were intraepithelial and clustered in subepithelial macrophages within 5 minutes of contact. However, as in most other previous assessments of uptake by mucosal lymphoid tissues, we made no attempt at quantification or in determining age effects on uptake. The objectives of the present study were to use computer-assisted measurements

to assess tracer uptake by CALT in chickens and to statistically analyze effects due to age, tracer contact time, and tracer particle type.

MATERIALS AND METHODS

Eighty-two 1-day-old broiler chickens were obtained from a local hatchery (Hoover Hatchery, Rudd, Iowa) after debeaking and subcutaneous administration of a killed Marek's disease virus vaccine in the dorsal cervical region. Chickens were housed in a well-ventilated room in an indoor laboratory animal facility concurrently housing no other chickens. After 2 weeks in temperature-controlled brooders, chickens were moved to wire cages for 3 weeks and then placed on concrete flooring with sawdust litter until the end of the experiment. Free access to fresh water and feed was provided at all times.

Tracer particle uptake was quantitatively assessed using two tracers (carbon and iron) in chickens of four ages (1, 3, 5, and 7 weeks) at three contact times (5, 15, and 30 minutes). Three chickens were used for each treatment combination of age, contact time, and tracer type. Computer-generated morphologic measurement of lower eyelid CALT nonLE and LE was performed in all of the above chickens and in additional 1-day-old (n = 5) and 16-week-old (n = 5) chickens.

Tracer particle suspensions of carbon and iron oxide were prepared to assess uptake by CALT in the lower eyelid. For carbon, 1 ml of black India Ink (Faber-Castell Corp., Newark, New Jersey) was diluted in 20 ml phosphate buffered saline (PBS, pH 7.2) and centrifuged at 5,000 x g for 30 minutes. After discarding the supernatant, the pellet was resuspended in 10 ml PBS. For iron, 100 mg of black iron oxide particles (0.5 μm diameter, Polysciences, Inc., Warrington, Pennsylvania) was suspended in 10 ml PBS. Tracer solutions were warmed to approximately 38 C prior to use.

At 1, 3, 5, and 7 weeks of age, 18 chickens were anesthetized with sodium pentobarbital and eyelid margins were sealed with adhesive bilaterally (Duro Superglue-5, Woodhill Permatex, Cleveland, Ohio). Each naso-lacrimal duct was

ligated with a single subcutaneous suture anterior to the nasal palpebral commissure to seal the conjunctival space. One ml of each tracer suspension was injected by syringe (22 gauge needle) through the right upper eyelid into the sealed conjunctival space of 9 chickens in each age group. Sufficient tracer suspension was injected to ensure adequate contact with CALT. For controls, PBS without tracer was injected into the left sealed conjunctival space of each bird.

Within each treatment combination of age and tracer type, 3 chickens were euthanatized with sodium pentobarbital after 5, 15, or 30 minutes of contact time. Lower eyelids were removed and fixed in 10% neutral buffered formalin. After 24 hours, the middle of each eyelid was trimmed in cross-section perpendicular to the eyelid margin. Eyelids were processed by routine paraffin technique after graded alcohol dehydration. Twenty-five serial sections were cut at 5 μm intervals from each block and divided into 5 consecutive groups. Within each group, the first section was stained with hematoxylin and eosin (HE), the second section was stained with fast green (FG), and the remaining 3 sections were discarded. Microscopic assessment of tracer particle uptake was restricted to lymphoepithelium (LE) and nonlymphoepithelium (nonLE) in proximal conjunctival folds and fissures of the lower eyelid (Fig. 1).

A measurement (in mm) of the surface contour distance along CALT nonLE and LE was determined in each section with a computerized image analysis system (Zeiss SEM-IPS Image Analysis System, Zeiss-Kontron;IBAS Version 1.3, Thornwood, New York). Using the HE sections described above, conjunctival folds and fissures containing CALT were imaged with computer digitalization under 2.0 x magnification (Fig. 1). The eyelid image was discriminated in shades of gray and the surface contour was traced. This tracing converted the eyelid surface contour to a single line representing the

linear surface distance (Fig. 2). By simultaneously comparing the computer-generated line with the actual tissue section (performed by A. S. Fix), regions of LE and nonLE were differentiated and the single line tracing was cut appropriately (Fig. 2, dotted lines). The computer then generated a numerical surface contour distance in mm for nonLE and LE in each section. To assess trends in CALT development with age, the surface contour distance in mm was converted to percent nonLE and LE in each section. Preliminary studies comparing the first and last sections from a given block revealed no significant differences in surface contour distance. Therefore, only the middle HE section from each block was measured by computer.

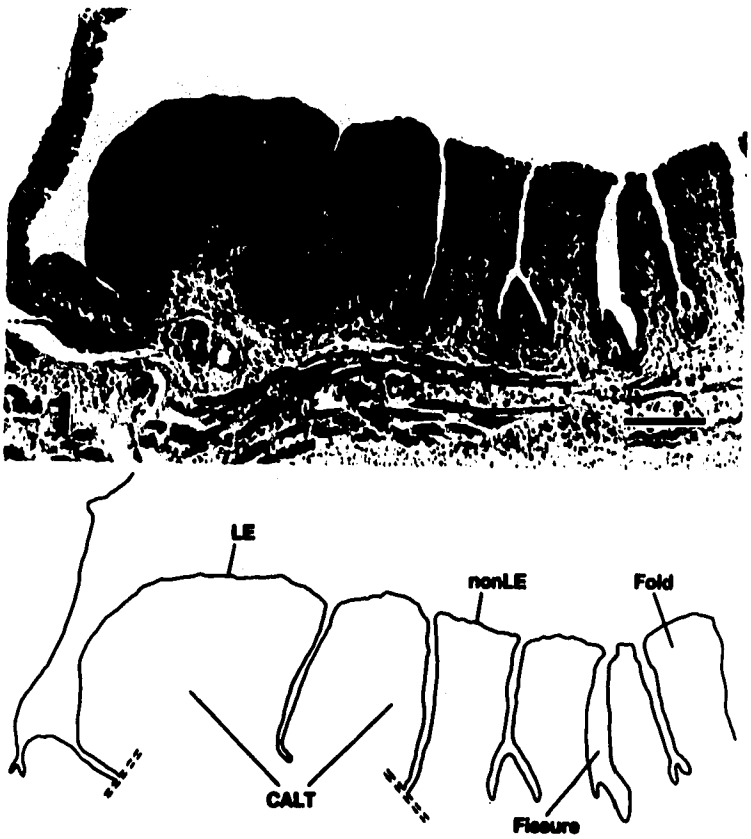
All FG sections were examined by light microscopy, and tracer particle uptake was scored numerically (performed by A. S. Fix) for each site of uptake as follows: 1 = individual tracer particle, 2 = small cluster of less than 10 particles, 3 = large cluster of greater than 10 particles (Fig. 3). Tracer particles had to clearly be within or below the superficial epithelium in order to be counted. Uptake was scored separately for nonLE and LE within each eyelid.

An uptake index was generated for both nonLE and LE in each section examined by dividing the uptake score by the respective surface contour distance in mm. This adjusted the numerical uptake score for the variability in CALT between sections. Uptake indices were analyzed by analysis of variance (ANOVA) using the general linear models procedure (PROC GLM) with SAS software (Statistical Analysis System Institute, Inc., Cary, North Carolina). Data were considered in a factorial experimental design with 24 treatments (4 ages x 3 contact times x 2 tracers). To assess morphologic changes in CALT with age, additional ANOVA was performed on computer-generated surface distance data.

Fig. 1: Proximal conjunctival folds and fissures, conjunctiva-associated lymphoid tissue, lower eyelid, 3-week-old chicken. Note distention of left two folds by lymphoid nodules. HE. Bar = 1 mm

Fig. 2: Computer-generated tracing of surface contour from Fig. 1. Dotted lines indicate demarcation of lymphoepithelium (LE) from nonlymphoepithelium (nonLE) bordering conjunctiva-associated lymphoid tissue (CALT)

Fig. 3: Subepithelial clusters of carbon tracer below the lymphoepithelium of conjunctiva-associated lymphoid tissue, 3-week-old chicken. Uptake was scored based on particle density at each site of uptake: individual particles = 1 (not shown); small clusters of <10 particles = 2 (arrowhead); larger clusters of >10 particles = 3 (arrow). FG. Bar = 10 μm



2

3

RESULTS

A significant increase in the lymphoepithelium (LE) surface contour distance existed in proximal conjunctival folds between all ages examined ($P = 0.05$). Significant differences were detected for measurements in mm and percentage LE (Fig. 4). Increases in percentage LE occurred by 3 weeks of age and over 80% of proximal conjunctival epithelium was LE by 7 weeks of age. Chickens 16 weeks of age had 100% LE in proximal conjunctival folds.

Carbon and iron tracers were observed primarily within and below the LE of CALT along proximal conjunctival folds in fast green (FG) sections (Fig. 3). Tracer particles were intraepithelial as well as in subepithelial clusters, probably within macrophages. The localization of carbon particles was more consistent than for iron particles, since some surface-adherent iron was displaced into deeper tissue by sectioning. The consistency of tracer localization in CALT was verified by examination of adjacent hematoxylin and eosin (HE) and FG sections.

The uptake index for LE was significantly greater than for nonlymphoepithelium (nonLE) when averaged over all treatment groups ($P = 0.01$). Within LE, significant differences were detected in uptake indices for age, contact time, and tracer type (Table 1). The mean uptake index increased significantly between 3 and 5 weeks of age and 5 and 15 minutes of contact time. Carbon produced a significantly higher mean uptake index in LE than iron. When iron tracer was excluded from statistical analyses and only carbon data were assessed, the differences detected for age and time were similar as described above and had a higher level of significance.

Fig. 4: Percentage of conjunctival folds and fissures occupied by chicken conjunctiva-associated lymphoid tissue (CALT) lymphoepithelium (LE) and nonlymphoepithelium (nonLE) at specific weeks post-hatching. LE = black; nonLE = light gray; age 0 = 1 day post-hatching. Percentages represent computer-generated measurements of LE and nonLE surface contour distance in histologic cross section

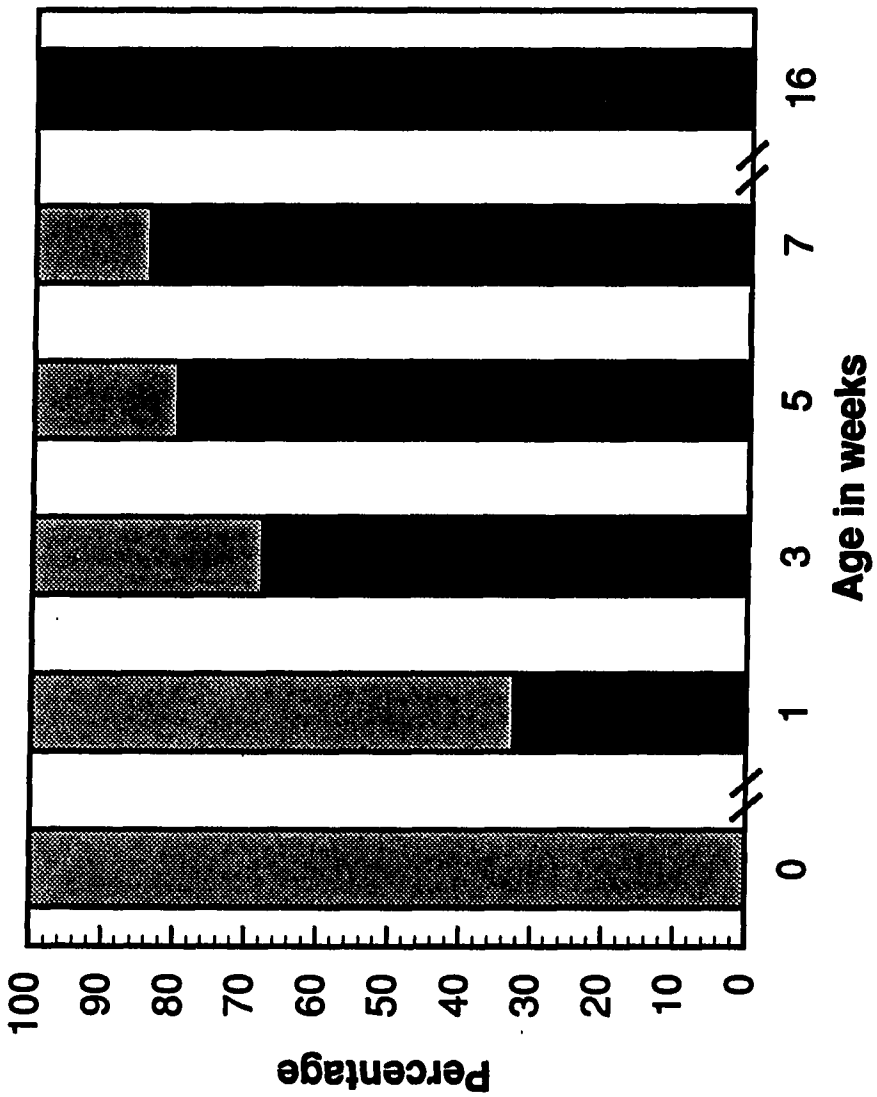

Percentage
100
90
80
70
60
50
40
30
20
10
0
Age in weeks
0
1
3
5
7
16

Table 1. Effects of age, contact time, and tracer type on particle uptake by conjunctiva-associated lymphoid tissue in chickens

	Treatment Group[A]	Uptake Index[B]
Age	1 week	1.3[a]
	3 weeks	1.6[a]
	5 weeks	6.1[b]
	7 weeks	6.2[b]
Contact time	5 minutes	2.3[a]
	15 minutes	4.6[b]
	30 minutes	4.6[b]
Tracer	carbon	5.3[a]
	iron	2.3[b]

[A]Statistical comparisons valid only within each treatment group.

[B]Mean uptake index. Values within each treatment group followed by different letters are significantly different ($P < 0.05$). Standard error of the mean for each treatment group: age = 0.29, contact time = 0.26, tracer = 0.21.

DISCUSSION

This study confirmed that conjunctiva-associated lymphoid tissue (CALT) in chickens undergoes an age-dependent increase in size and that the uptake of particulate material within the conjunctival space occurs through CALT. Uptake occurs primarily through lymphoepithelium (LE), increases between 3 and 5 weeks of age, and is quite rapid. These data support the idea that CALT is a functional component of the mucosal immune system and that through the uptake of antigen, CALT may have a role in paraocular and upper respiratory immunity of chickens.[72]

The qualitative characteristics of carbon and iron uptake in these chickens closely resembles the uptake of the same tracers in turkeys.[73] In both species, post-uptake localization occurred in the folds of CALT in both intraepithelial and subepithelial sites. Although multiple ages were not evaluated in the turkeys of our previous study, uptake was extremely rapid and increased with contact time.[73] The similarity in the results of these two avian studies suggests common functional features of CALT in birds. However, a similar study assessing the uptake of horseradish peroxidase by CALT in guinea pigs failed to demonstrate selective localization in LE.[178] The significance of this species difference is currently unknown.

The quantitative, age-dependent increase in the size of LE detected by our computer measurements is consistent with previous qualitative observations of CALT in chickens[72] and turkeys.[71] In both species, lower eyelid CALT is not present at hatching but is prominent and contains germinal centers by 2 to 3 weeks of age.[71,72] Similarly, CALT in rabbits is not evident until after 4 weeks of age.[13] The structure of CALT in rabbits, turkeys, and chickens[13,71,72] is comparable to other mucosal lymphoid tissues.[24] Therefore, the location and

accessibility of CALT makes it a valuable model for study of postnatal development of mucosal lymphoid tissues.

The morphologic change from proximal conjunctival nonlymphoepithelium (nonLE) to LE correlates with the increased capacity for particle uptake detected in older chickens. As chickens age, the nonLE, which is composed of tall columnar epithelial cells and goblet cells, changes to a more flattened LE lacking goblet cells but containing intraepithelial lymphocytes.[72] This epithelial change probably promotes the capacity for particle uptake, which occurs through specialized epithelial cells in mucosal lymphoid tissues.[202] Most studies assessing uptake in mucosal lymphoid tissues have examined post-neonatal animals, usually of one age.[140,174,183] Our approach was unique in evaluating and correlating the simultaneous structural and functional development of CALT from hatching to 7 weeks of age. Although the only significant morphologic change in chicken CALT between 3 and 6 weeks of age is the appearance of plasma cells,[72] our data suggest that uptake increases during this period. Therefore, animal age should be strongly considered when planning and interpreting uptake studies involving mucosal lymphoid tissues.

Tracer localization within or below the CALT LE in the present study was remarkably rapid, suggesting that minimal contact time is necessary to trigger uptake. As with carbon in the bursa of Fabricius[130] and horseradish peroxidase in GALT,[140] we found uptake occurring within 5 minutes of contact time. If similarly fast uptake occurs under field conditions, then a mechanism exists for rapid sampling of potentially antigenic materials located within the conjunctival space. This characteristic is highly advantageous if vaccination strategies are to be developed for specific immunization through CALT.

Although we have demonstrated that uptake of tracer particles occurs through CALT in chickens experimentally, direct evidence that this occurs with antigens delivered under field conditions is lacking. However, CALT does undergo lymphoid hyperplasia in turkeys infected with Bordetella avium,[71] as has been described for BALT in similarly infected turkeys.[191] In addition, evidence suggests that eyedrop delivery of antigen results in increased plasma cell infiltration into and specific antibody response by the chicken Harderian gland.[41,61,153,181] We therefore consider it likely that CALT functions in antigen uptake and has a role in the mucosal immune system of chickens. In addition, CALT may have an age-specific maturity which correlates with this uptake function.

GENERAL SUMMARY

These studies have described the gross, histologic, and ultrastructural features of conjunctiva-associated lymphoid tissue (CALT) in turkeys and chickens. In addition, the uptake of particulate tracer material by CALT has been documented in both species. This work represents the first descriptive and experimental assessment of CALT in birds. Overall, CALT in turkeys and chickens is quite similar in location and structure to that described in rabbits and guinea pigs.[13,47,78] However, studies concerning the function of CALT in mammals have been limited.[85,178] In contrast to the present avian studies, functional evaluation of CALT in mammals failed to document selective tracer uptake by CALT lymphoepithelium.[178]

CALT in turkeys and chickens has many features found in mucosal lymphoid tissues. In these avian studies, CALT was absent at hatching and progressively developed during the post-hatching period. Within one week, lymphoid nodules were evident within the proximal conjunctival longitudinal folds and fissures in the lower eyelid of both species. By three weeks of age, lymphoid nodules were quite prominent and occupied a large portion of the folds and fissures. Computerized image analysis documented the statistical significance of these age-related changes in chicken CALT. Many epithelial and vascular structural features found in mucosal lymphoid tissues[24,146,202,204] were evident. The superficial epithelial cells that formed the lymphoepithelium were flattened, had prominent intercellular junctions, contained apical vesicles, and had short, irregular microvilli. Within the lymphoepithelium, intraepithelial lymphocytes, a lack of goblet cells, and a discontinuous epithelial basement membrane were also characteristic. Vessels associated with CALT, particularly those at the base

of lymphoid nodules, had high endothelial cells and adherent intraluminal lymphocytes. Germinal centers and lymphoid hyperplasia associated with Bordetella avium infection in turkeys indicated the reactivity of CALT to local antigenic stimulation. Collectively, similarities between avian CALT and other mucosal lymphoid tissues strongly suggest a role for CALT in mucosal immunity.

The uptake of tracer particles documented by these studies further supports CALT as a component of the mucosal immune system. Mucosal lymphoid tissues have the unique ability to sample mucosal surfaces by the transepithelial uptake of particulate material.[22,202] In the present studies, carbon, iron oxide, and latex beads were selectively taken up across the CALT lymphoepithelium in turkeys. Clustering within subepithelial macrophages was characteristic after uptake. Tracer uptake also increased with contact time and was more extensive in older birds. In addition, the site of tracer uptake varied with particle size, occurring superficially along lymphoepithelial folds for smaller particles but deeper within fissures for larger particles. These qualitative features of tracer uptake in turkeys were substantiated by computerized image analysis and quantitative studies in chickens. A statistically significant increase in uptake was demonstrated between 5 and 15 minutes of tracer contact time and between 3 and 5 weeks of age.

The results of these studies offer a unique hypothesis concerning paraocular immunity in birds, particularly when interpreted in light of the preexisting literature on the avian Harderian gland. Since CALT in turkeys and chickens has structural features consistent with mucosal lymphoid tissues and the uptake of tracer material has been documented experimentally, a role in mucosal immunity is quite likely. The avian Harderian gland contains predominantly plasma cells and secretes IgA.[5,64,180] These experiments indicate that CALT

could provide an initial site of antigen uptake; subsequent processing and presentation of antigen may lead to an antibody response that provides mucosal protection. Localization of plasma cells to the Harderian gland or to other local epithelial tissues may provide a homing-loop mechanism specific for protection against upper respiratory pathogens.

The extent to which CALT functions as a mucosal lymphoid tissue under natural circumstances is entirely unknown. A number of vaccines for important avian pathogens are administered by mucosal routes. These include aerosolized delivery for respiratory contact, oral delivery for intestinal contact, and eyedrop delivery for ocular contact. Based on simultaneous exposure of large numbers of birds, these delivery methods could easily provide vaccine contact with conjunctival lymphoid tissue. Specific studies examining the role of CALT in immune responsiveness to these vaccines are necessary.

Given the age-dependent development and maturation that occurs in avian CALT, vaccination protocols used in the poultry industry may require reassessment. For example, some vaccination programs begin with eyedrop delivery of vaccine in day-old chicks. Based on the present studies, which indicate a complete lack of lymphoid tissue in the conjunctiva at one day of age, the usefulness of this technique may be questionable. Delay of vaccination until CALT is functionally more mature would appear more appropriate. However, a specific maturation time has not been determined and may be influenced by numerous factors including species, breed, strain, nutritional status, and local antigenic stimulation. In addition, the role CALT may have in response to vaccines may differ for killed and modified-live products.

Many important questions remain to be answered concerning the role of CALT in avian mucosal immunity. Specific mechanisms of transepithelial tracer uptake require assessment

at the electron microscopic level. The temporal appearance and isotype specificity of plasma cells in CALT should be evaluated. The localization of lymphocytes originating in CALT and the potential homing of those lymphocytes to the Harderian gland are completely unexplored. Information gained from further research in these areas will enhance our understanding of conjunctiva-associated lymphoid tissue in birds and may improve our control of economically significant avian diseases.

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